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Carbonyl- and sulfur-containing analogs of suberoylanilide hydroxamic acid: Potent inhibition of histone deacetylases

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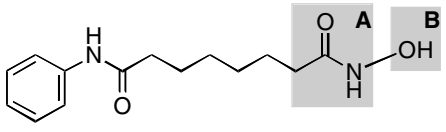
Abstract—Suberoylanilide hydroxamic acid (SAHA), an inhibitor of histone deacetylase, is used in clinical trials for a variety of advanced cancers. Twelve new analogs of SAHA were synthesized and tested as in vitro inhibitors of isolated histone deacetylases (HDACS) and in vivo inhibitors of interferon regulated transcriptional responses (a marker for HDAC activity). The analogs containing an α-mercaptoketone or an α-thioacetoxyketone were more potent than SAHA in both assays.

1. Introduction

Epigenetic regulation of gene expression is mediated by several mechanisms, including DNA methylation, ATP-dependent chromatin remodeling, and post-]translational modifications of histones, such as methylation, phosphorylation, ubiquitinylation, or acetylation. Dynamic acetylation of ε-amino groups of lysine residues on core histone tails is regulated by two opposing enzymes, histone acetyltransferase (HAT) and histone deacetylase (HDAC).[1] Transcriptional regulation is controlled by HAT enzymes, which add acetyl groups to lysine residues of histones and non-histone proteins, and HDACs, which remove these modifications, thereby mediating chromatin remodeling and gene expression.[2] Pharmacological HDAC inhibitors comprise a novel class of cancer chemotherapeutics in clinical development that target HDAC enzymatic activity thereby inducing hyperacetylation of HDAC substrates, which results in altered chromatin structure and gene expression, and has been shown to induce growth arrest, cell differentiation, and apoptosis of tumor cells.[3] Furthermore, they suppress cell proliferation in a variety of transformed cells in culture and in tumor, bearing animals, and have shown great promise as new anticancer drugs.[4] Several structurally diverse HDAC inhibitors now in clinical trials have demonstrated encouraging antitumor activity in a variety of cancer types, enhancing the rationale for continued development of new HDAC inhibitors.[5]

Suberoylanilide hydroxamic acid (SAHA) (Fig. 1) is one of the early HDAC inhibitors,[6] which inhibits cell growth, induces terminal differentiation in tumor cells,[7] prevents the formation of malignant tumors in mice,[8] and is currently in phase III clinical trials.[9] Crystal structures of a hyperthermophilic bacterium HDAC[10] and of human HDAC8[11] with SAHA bound show that the



Suberoylanilide Hydroxamic Acid (SAHA)

Figure 1. Structure of suberoylanilide hydroxamic acid (SAHA).

Abbreviations: HDAC, histone deacetylase; SAHA, suberoylanilide hydroxamic acid; HAT, histone acetyltransferase; ACE, angiotensin-converting enzyme; IFN, interferon; ISG, IFN-stimulated gene; TSA, trichostatin A.

Keywords: Histone deacetylase; Inhibitors; α-Mercaptoketone; Suberoylanilide hydroxamic acid; SAHA; α-Thioacetoxyketone.

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hydroxamic acid moiety coordinates to the active-site zinc ion. Hydroxamates, however, have been found to exhibit unfavorable pharmacokinetic behavior resulting from glucuronidation and sulfation,[12] and from metabolic hydrolysis,[13] all of which result in short in vivo half-lives of the hydroxamic acid group. Consequently, new potent inhibitors have been sought. Non-hydroxamate HDAC inhibitors discovered to date, however, have reduced potency compared to hydroxamates and have not shown any metabolic advantage.[14]

Although hydroxamates bind to zinc ions, they are not necessarily the optimal coordinators. Sulfur ligands are well known to bind tightly to zinc-containing enzymes.[15] This was clearly demonstrated by the invention of captopril, a thiol-containing inhibitor of the zinc-dependent enzyme angiotensin-converting enzyme (ACE).[16]

We have designed a series of SAHA analogs as potential inhibitors of HDACs, in which the hydroxamic acid was replaced by sulfur-containing moieties, which may have enhanced binding affinities for zinc. The optimal ligand from this series can be utilized as the preferred ligand for other HDAC inhibitor compounds.

2. Results and discussion

2.1. Chemistry

The hydroxamate group of SAHA was separated into two moieties: **A** and **B**, as shown in Figure 1, and a 12-membered library of SAHA analogs was designed (Fig. 2). The type of carbonyl moiety (**A**) was compared (Fig. 2, **1** vs **2**, **3**, **6**,and **11**). To compare the effectiveness of a sulfur atom versus an oxygen atom, compounds in which a sulfur or oxygen at the same position were synthesized (Fig. 2, **1** vs **2**; **3** vs **4** and **5**; **6** vs **7**; **12** vs **13**). The group at **B** was investigated as well (Fig. 2, **4** vs **9**; **7** vs **8** and **10**). While this research was being carried out, compounds **3**, **4**, and **9** were reported.[17]

SAHA was synthesized as previously reported.[18] The syntheses of **2**–**13** are shown in Schemes 1–6. Although **3**, **4**, and **9** have already been reported, we accomplished their syntheses by different routes. The synthesis of **2** was carried out by treating SAHA directly with Lawesson's reagent in THF at room temperature. Surprisingly, the expected target molecule **2** was obtained as a single product. The regioselectivity between the amide carbonyl and the hydroxamic carbonyl groups presumably derived from the hydroxamate hydroxyl group, which may form a strong bond with the phosphorus atom in Lawesson's reagent, favoring that carbonyl group (Scheme 2).

Compounds **6**, **7**, **8**, and **10** were synthesized from common intermediate **12**, which was tested as an inhibitor of HDACs as well. As shown in Scheme 3, compound **14**, which was prepared from 8-bromo-1-octene,[19] was treated with DIC and HOBt, and then coupled with aniline to afford **15**. The double bond of **15** was oxidized with *m*CPBA to give epoxide **12**, which was treated with potassium bromide in acetic acid and water to afford **16**. Compound **16** was oxidized with Jones reagent to

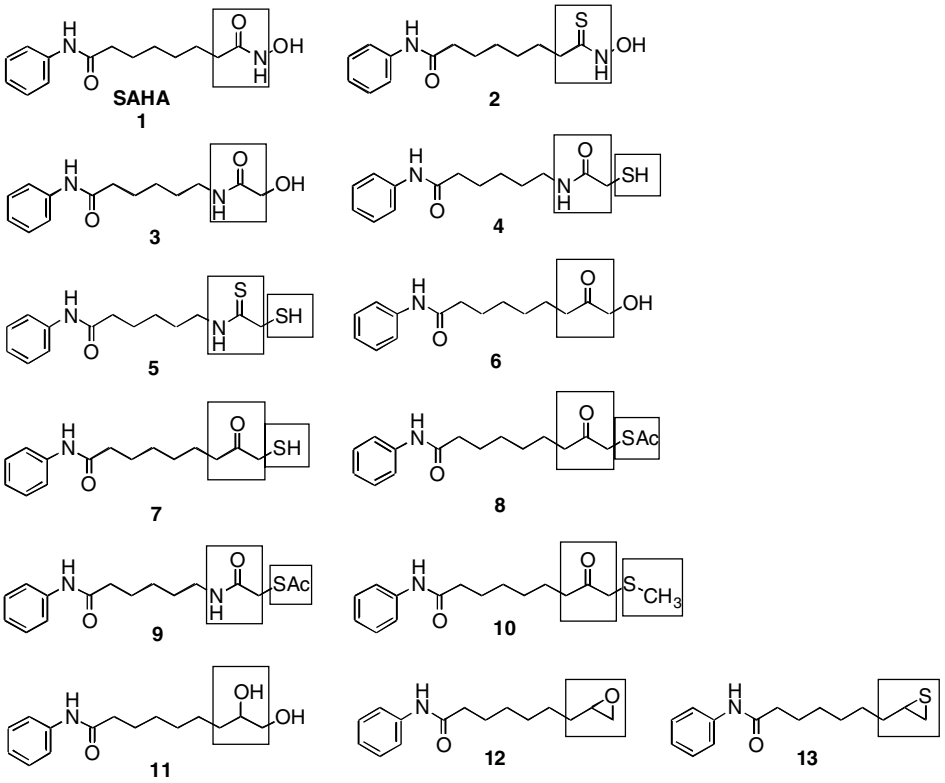
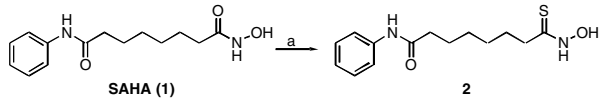
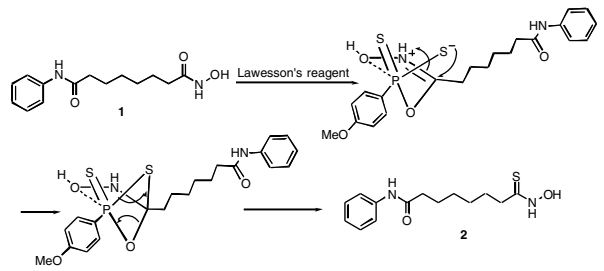


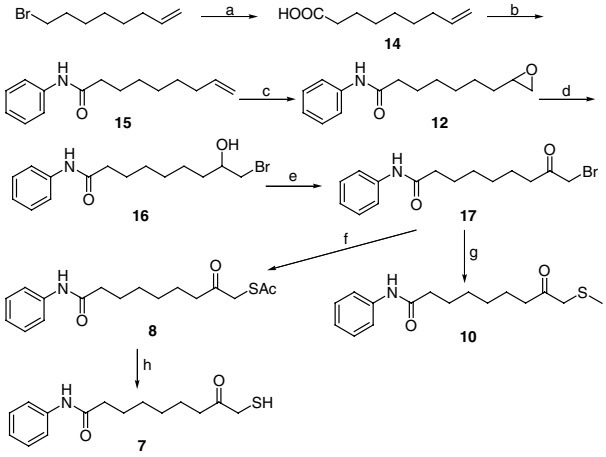
Figure 2. SAHA analogs synthesized.



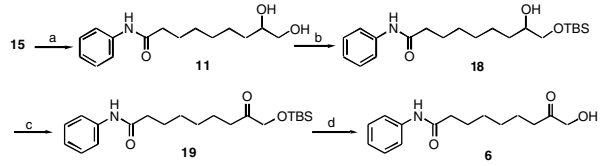
Scheme 1. Reagents and condition: (a) Lawesson's reagent, THF, rt, 62%.



Scheme 2. Possible mechanism for regioselective thionation of SAHA.



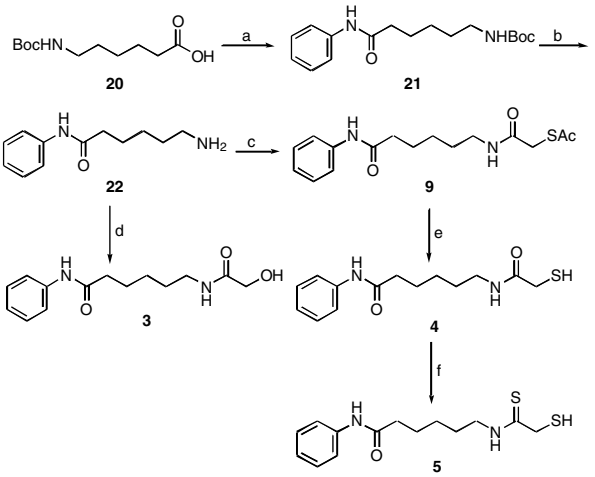
Scheme 3. Reagents and conditions: (a) two steps, 60%, Ref. 19; (b) aniline, DIC, HOBt, DIPEA, DMF, rt, 92%; (c) *m*CPBA, methylene chloride, rt, 85%; (d) KBr, AcOH, H_2O, rt, 71%; (e) Jones reagent, rt, 68%; (f) KSAc, DMF, rt, 86%; (g) $KSCH_3$, DMF, rt, 80%; (h) NaOH (aq, 3M)/acetone (1:1), rt, 83%.



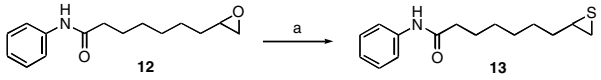
Scheme 4. Reagents and conditions: (a) OsO_4, NMO, $^tBuOH/H_2O$ (9:1), 0 °C to rt, 77%; (b) TBSCl, imidazole, DMF, rt, 90%; (c) $KMnO_4$, $CuSO_4{\cdot}H_2O$, benzene, rt, 52%; (d) TBAF, THF, rt, 89%.

generate α-bromoketone **17**. Potassium thioacetate was employed as the nucleophile to give **8**, which was then hydrolyzed to generate **7**. Compound **17** was treated with sodium thiomethoxide in DMF at room temperature to afford **10**.

Compounds **11** and **6** were synthesized via **15**. As shown in Scheme 4, **15** was oxidized with OsO_4 to generate **11**.



Scheme 5. Reagents and conditions: (a) aniline, DIC, HOBt, DIPEA, DMF, rt, 92%; (b) TFA, methylene chloride, rt, 99%; (c) AcS-CH_2COOH, PyBOP, DIPEA, DMF, rt, 88%; (d) glycolic acid, PyBOP, DIPEA, DMF/methylene chloride (1:9), rt, 80%; (e) NaOH (aq, 3M)/acetone (1:1), rt, 80%; (f) Lawesson's reagent, THF, rt, 57%.



Scheme 6. Reagents and condition: (a) thiourea, methanol, rt, 88%.

Several different oxidation conditions were attempted, but all of them failed to give the expected ketone **6** directly from **11**.[20] Compound **11** was then protected selectively with TBSCl to afford **18**, which was treated under mild oxidation conditions ($KMnO_4$, $CuSO_4{\cdot}H_2O$)[21] to generate **19**. Deprotection of **19** afforded **6**.

As shown in Scheme 5, compounds **3**, **4**, **5**, and **9** were synthesized from the same intermediate (**22**). Commercially available **20** was coupled with aniline using DIC and HOBt as the coupling reagents to afford **21**, which was deprotected to generate **22**. Compound **22** was coupled with acetyl thiolacetic acid using PyBOP as the coupling reagent to produce **9**. Hydrolysis of **9** gave **4**. Compound **4** was treated under the same conditions as was **2** using Lawesson's reagent, thereby producing the corresponding thiocarboxamide compound (**5**). Compound **22** also was converted to **3** in one step by coupling with glycolic acid. According to earlier work,[17] **3**, **4**, and **9** were synthesized in four steps, six steps, and five steps, respectively. The syntheses described here are each one step shorter. Scheme 6 shows the conversion of **12** to **13** by treatment with thiourea at room temperature.

2.2. Enzyme inhibition results

SAHA and its analogs were tested in vitro with commercially available HDAC assay kits, one for HeLa nuclear extract containing a mixture of HDACs and one with purified human recombinant HDAC8 (Table 1).[22]

It is apparent from Table 1 that **7** and **8** are more potent in vitro inhibitors of HDACs and of HDAC8 than is

Table 1. IC_{50} value[a] of analogs of SAHA against HDACs and HDAC8

Entry	Inhibitors	IC_{50} (μM)	
		(HDACs)	(HDAC8)
1	SAHA (**1**)	0.37	0.82
2	**2**	>10	>10
3	**3**	>40	>40
4	**4**	2.44	3.89
5	**5**	13.3	>40
6	**6**	>40	>40
7	**7**	0.15	0.69
8	**8**	0.081	0.19
9	**9**	20.1	>40
10	**10**	>40	>40
11	**11**	>40	>40
12	**12**	>40	>40
13	**13**	>40	>40

[a] Values are means of at least three experiments.

SAHA (**1**); others have reported a similar observation in other series of compounds.[23] The similarity in potencies of **7** and **8** is unexpected because thiols are strong ligands to Zn^{2+}, so **7** should have been more effective than **8**. Given the similar IC_{50} values for these two inhibitors, it was thought that possibly **8** was hydrolyzed to **7**. However, mass spectrometry after inhibition showed that **8** was intact and no **7** was formed. These results are different from those with **4** and **9**, which hold the same relationship as **7** and **8**, respectively. In this case, thioacetate **9** is considerably less potent than thiol **4**. Additional studies into these differences are underway.

In general, the potency of the compounds toward the mixture of HDACs is greater than that for HDAC8. A change in the hydroxamate to an α-hydroxyamide (**3**) or to an α-hydroxyketone (**6**) resulted in a sharp decrease in potency (>100-fold for HDACs or HDAC8); conversion to the corresponding thiohydroxamate (**2**) decreased the potency by >25 times. Reduction of the ketone carbonyl to an alcohol (**11**) also gave poor potency. Because of the superior inhibition of gelatinases (matrix metalloproteinases) by a thiirane relative to the corresponding oxirane,[24] thiirane **13** was prepared and compared to oxirane **12**. Both were found to be weak inhibitors of HDACs, too weak to make an accurate comparison.

The effect of modifying the **B** moiety (the α-hydroxyl group) also was dramatic. In the reverse amide series, replacement of the α-hydroxyl group of **3** with an α-mercapto group (**4**) resulted in a >15-fold increase in potency; conversion of the amide to a thioamide along with substitution of the α-hydroxyl group by an α-mercapto group (**5**) lowered the potency 5-6 fold relative to **4**. It is apparent that a thiol group is beneficial for binding, but conversion of the carbonyl oxygen to sulfur is detrimental to binding. Another example of this phenomenon is the conversion of the α-hydroxyl group of ketone **6** to an α-mercapto group (**7**), which results in about a 250-fold increase in potency. Because of the possible lability of the thiol group, modifications were made to it. As discussed above, if the thiol group of **4** is converted to a thioacetoxyl group (**9**), the potency decreases by a factor of about 8, but if the same modification is made to the corresponding α-mercaptoketone **7** to give the α-thioacetoxyketone **8**, there is an almost 2-fold increase in potency. It appears that the ketone carbonyl is synergistic in binding with the α-thioacetoxyl group. S-Methylation of α-mercaptoketone **7** to give α-methylthioketone **10** results in a >250-fold decrease in potency.

2.3. Effect of HDAC inhibitors on interferon transcriptional responses

Further biological tests were made in cell culture. Although HDAC activity has been conventionally associated with transcriptional repression, recent findings demonstrated that HDACs can function as transcriptional coactivators in the interferon (IFN) system.[25] In response to IFN, the IFN-stimulated gene (ISG) mRNAs rapidly and transiently accumulate in cells, but HDAC inhibitors such as trichostatin A (TSA), sodium butyrate, or SAHA prevent ISG transcription.[23a,b]

The ability of SAHA and its analogs to inhibit IFN transcriptional responses was tested. Human fibrosarcoma cell line 2fTGH was used in an IFN-dependent luciferase reporter gene assay in the absence or presence of HDAC inhibitor compounds (Fig. 3). In this assay, both the IFN-responsive luciferase construct and a control *Renilla* luciferase were transfected into cells, and cells were stimulated with IFN_α for 6 h prior to analysis. Compounds **7** and **8** were able to prevent IFN-inducible reporter gene activity, in agreement with their abilities to function as general HDAC inhibitors.

3. Conclusions

An α-mercaptoketone and α-thioacetoxylketone are improved zinc-binding ligands relative to hydroxamate in

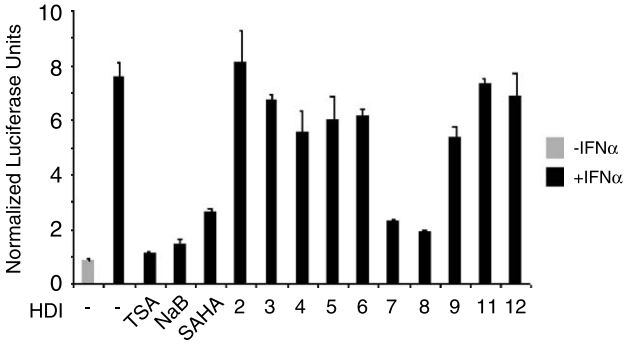


Figure 3. Inhibition of IFN-induced reporter gene expression by SAHA derivatives. Luciferase assays were performed in human fibrosarcoma 2fTGH cells using an IFN-dependent luciferase reporter and *Renilla* luciferase for normalization. Cells were treated with IFN_α and simultaneous HDAC inhibitors (HDI) as shown.

this study and should be valuable groups to attach to molecules that target not only histone deacetylase, but other zinc-dependent enzymes.

4. Experimental

Conventional organic solvents were purchased from Fisher. All of the reagents were purchased from Aldrich Chemical Co. and were used without further purification unless stated otherwise. Methylene chloride was distilled under N_2 from calcium hydride. Flash chromatography was performed with Merck silica gel (230–400 mesh). TLC plates (silica gel 60-F254) were purchased from VWR Scientific. All ^{1}H NMR spectra were recorded on Varian Gemini 300 MHz, Mercury 400, or Inova 500 spectrometers (75, 100, or 125 MHz for ^{13}C NMR spectra). Chemical shifts (δ) are reported downfield from tetramethylsilane (Me_4Si) in parts per million (ppm). Compounds were visualized with a ninhydrin spray reagent or a UV/vis lamp. Mass spectra were recorded either on a VG Instrument VG70-250SE high-resolution mass spectrometer (ESI) or on a Micromass Quattro II spectrometer (APCI).

4.1. Suberoylanilide hydroxamic acid (1)

Compound **1** (SAHA) was synthesized by a literature method.[†] ^{1}H NMR (DMSO-d_6): δ 1.27 (s, 4H), 1.48 (t, J = 6.0 Hz, 2H), 1.56 (br s, 2H), 1.94 (t, J = 7.2 Hz, 2H), 2.28 (t, J = 7.2 Hz, 2H), 7.01 (t, J = 7.2 Hz, 1H), 7.27 (t, J = 7.6 Hz, 2H), 7.58 (d, J = 7.6 Hz, 2H), 8.67 (s, 1H), 9.86 (s, 1H), 10.34 (s, 1H); ^{13}C NMR (DMSO-d_6): δ 25.1, 28.4, 32.3, 36.4, 38.9, 119.0, 122.9, 128.6, 139.4, 169.1, 171.2. EI-MS (M^+): 264.2, 248.2, 230.2, 190.2, 148.1, 135.1, 93.1. HRMS Calcd for $C_{14}H_{20}N_2O_3$ 264.1468, found 264.1466. Anal. Calcd for $C_{14}H_{20}N_2O_3{\cdot}0.05H_2O$: C, 63.40; H, 7.64; N, 10.56. Found: C, 63.14; H, 7.67; N, 10.27.

4.2. Thiosuberoylanilide hydroxamic acid (2)

Compound **1** (SAHA) (0.26 g, 1.00 mmol) was dissolved in anhydrous THF (5 mL) followed by the addition of 2,4-bis(4-methoxyphenyl)-1,3-dithia-2,4-diphosphetane-2,4-disulfide (Lawesson's reagent, 0.20 g, 0.50 mmol) portionwise. The reaction mixture was covered with aluminum foil and stirred for 4 h at room temperature. The solvent was removed under vacuum, and the residue was purified by flash chromatography eluting with hexane/EtOAc (1:1) to give the desired thiosuberoylanilide hydroxamic acid **2** (174 mg, 62%). ^{1}H NMR (CD_3OD): δ 1.40 (s, 4H), 1.70 (m, 4H), 2.36 (t, J = 7.2 Hz, 2H), 2.58 (t, J = 7.2 Hz, 2H), 7.06 (t, J = 7.2 Hz, 1H), 7.28 (t, J = 8.0 Hz, 2H), 7.52 (d, J = 7.6 Hz, 2H); CI-HRMS Calcd for $C_{14}H_{20}N_2O_2S$ 281.1318, found 281.1317. Anal. Calcd for $C_{14}H_{20}N_2O_2S{\cdot}0.2H_2O$: C, 59.21; H, 7.24; N, 9.86; S, 11.29. Found: C, 59.23; H, 6.96; N, 9.72; S, 11.16.

[†] Stowell, J. C.; Huot, R. I.; Voast L. V. The synthesis of *N*-hydroxy-*N*′-phenyloctanediamide and its inhibitory effect on proliferation of AXC rat prostate cancer cells. *J. Med. Chem.* **1995**, *38*, 1411–1413.

4.3. 6-(2-Hydroxyacetylamino)-*N*-phenylhexanamide (3)

To a stirred solution of glycolic acid (114 mg, 1.5 mmol) in a mixed solvent system of anhydrous DMF and anhydrous methylene chloride (1:9, 5 mL) were added PyBOP (781 mg, 1.5 mmol) and DIPEA (0.78 mL, 4.5 mmol) at room temperature. Then **22** hydrochloric acid salt (365 mg, 1.5 mmol) was added to the reaction mixture in one portion. The reaction mixture was stirred overnight. The solvent was removed under vacuum, and the residue was dissolved in ethyl acetate. The organic phase was washed with 0.5 N HCl solution, saturated $NaHCO_3$, and brine. The organic phase was then dried over Na_2SO_4 and evaporated in vacuo. The crude product was recrystallized from EtOH/hexanes to give **2** (317 mg, 80%). ^{1}H NMR (CD_3OD): δ 1.42 (t, J = 8.0 Hz, 2H), 1.59 (p, J = 7.1 Hz, 2H), 1.73 (p, J = 7.2 Hz, 2H), 2.38 (t, J = 7.2 Hz, 2H), 3.26 (t, J = 7.2 Hz, 2H), 3.95 (s, 2H), 7.08 (t, J = 7.6 Hz, 1H), 7.29 (t, J = 8.0 Hz, 2H), 7.54 (d, J = 8.4 Hz, 2H); ^{13}C NMR (CD_3OD): δ 26.7, 27.7, 30.4, 37.9, 39.9, 48.5, 62.7, 121.4, 125.3, 129.9, 140.0, 174.6, 175.3. EI-MS (M^+): 264.2, 233.1, 135.1, 93.1. HRMS Calcd for $C_{14}H_{20}N_2O_3$ 264.1468, found 264.1464. Anal. Calcd for $C_{14}H_{20}N_2O_3{\cdot}0.2H_2O$: C, 62.76; H, 7.67; N, 10.46. Found: C, 62.89; H, 7.62; N, 10.31.

4.4. 6-(2-Mercaptoacetylamino)-*N*-phenylhexanamide (4)

Reagent grade acetone (5 mL) and 3 N NaOH (5 mL) in a 25 mL flame-dried flask were deoxygenated with argon for 20 min at 0 °C. Under positive Ar pressure, the crude compound **9** (32 mg, 0.1 mmol) was added as a solid to the flask. This reaction mixture was stirred under Ar for 6 h. A solution of 1 M HCl was added to neutralize the solution. The aqueous fraction was extracted three times with ethyl acetate. The combined extracts were washed with brine (10 mL), dried over Na_2SO_4, and evaporated in vacuo, and the residue was purified by flash chromatography eluting with EtOAc followed by recrystallization from 95% EtOH to give **4** (22 mg, 80%) as a white solid: ^{1}H NMR (CD_3OD): δ 1.44 (d, J = 7.1 Hz, 2H), 1.57 (p, J = 7.2 Hz, 2H), 1.72 (p, J = 7.0 Hz, 2H), 2.38 (t, J = 7.2 Hz, 2H), 3.12 (s, 2H), 3.20 (t, J = 6.6 Hz, 2H), 7.08 (t, J = 7.2 Hz, 1H), 7.29 (t, J = 7.6 Hz, 2H), 7.54 (d, J = 7.6 Hz, 2H), 8.11 (s, 1H); ^{13}C NMR ($CDCl_3$): δ 26.7, 27.6, 28.4, 30.2, 37.9, 40.7, 121.4, 125.2, 129.9, 140.0, 173.4, 174.6. EI-MS (M^+): 280.1, 248.2, 207.2, 188.1, 178.1, 135.1, 114.1, 93.1. HRMS Calcd for $C_{14}H_{20}N_2O_2S$ 280.1240, found 280.1236. Anal. Calcd for $C_{14}H_{20}N_2O_2S{\cdot}0.2H_2O$: C, 59.21; H, 7.24; N, 9.86; S, 11.29. Found: C, 59.23; H, 6.96; N, 9.72; S, 11.16.

4.5. 6-(2-Mercapto[thioacetylamino])-*N*-phenylhexanamide (5)

Compound **4** (28 mg, 0.1 mmol) was dissolved in anhydrous THF (2 mL) followed by the portionwise addition of 2,4-bis(4-methoxyphenyl)-1,3-dithia-2,4-diphosphetane-2,4-disulfide (Lawesson's reagent, 20 g, 0.05 mmol). The reaction mixture was covered with aluminum foil and stirred for 4 h at room temperature. The solvent was removed under vacuum, and the residue

was purified by flash chromatography eluting with hexane/EtOAc (1:1) to give **5** (16.9 mg, 57%). ^{1}H NMR (CD_3OD): δ 1.42 (p, J = 7.0 Hz, 2H), 1.56 (p, J = 7.0 Hz, 2H), 1.71 (p, J = 7.0 Hz, 2H), 2.36 (t, J = 7.0 Hz, 2H), 3.22 (t, J = 7.0 Hz, 2H), 3.29 (s, 2H), 6.92 (d, 9.0 Hz), 7.06 (t, J = 7.0 Hz, 1H), 7.27 (t, J = 7.5 Hz, 2H), 7.52 (d, J = 7.5 Hz, 2H), 7.86 (m, 1H). ESI-MS (M^++1): 297.2, 207.1, 188.1, 190.1, 135.1, 114.1, 93.1.

4.6. 9-Hydroxy-8-oxo-*N*-phenylnonanamide (6)

To a solution of **19** (10 mg, 0.027 mmol) in 0.5 mL THF was added TBAF (0.05 mL, 1.0 M in THF, 0.05 mmol) at room temperature. The reaction was monitored by TLC until it was complete. The reaction mixture was then evaporated in vacuo, and the residue was purified by flash chromatography eluting with hexane/EtOAc (1:1) to give **6** (6.3 mg, 89%). ^{1}H NMR ($CDCl_3$): δ 1.39 (br s, 4H), 1.67 (p, J = 7.2 Hz, 2H), 1.75 (t, J = 7.2 Hz, 2H), 2.36 (t, J = 7.2 Hz, 2H), 2.43 (t, J = 8.0 Hz, 2H), 3.09 (br s, 1H), 4.24 (d, J = 3.6 Hz), 7.11 (t, J = 7.2 Hz, 1H), 7.33 (t, J = 8.0 Hz, 2H), 7.43 (s, 1H), 7.52 (d, J = 8.0 Hz, 2H); ^{13}C NMR ($CDCl_3$): δ 23.6, 25.4, 27.0, 29.0, 37.8, 38.5, 68.3, 119.9, 124.5, 129.3, 171.3, 210.0. EI-MS (M^+): 263.2, 232.0, 135.1, 93.1. HRMS Calcd for $C_{15}H_{21}NO_3$ 263.1516, found 263.1513.

4.7. 9-Mercapto-8-oxo-*N*-phenylnonanamide (7)

Reagent grade acetone (5 mL) and 3 N NaOH (5 mL) in a 25 mL flame-dried flask were deoxygenated with argon for 20 min at 0 °C. Under positive Ar pressure, crude **8** (32 mg, 0.1 mmol) was added to the flask as a solid. This reaction mixture was stirred under Ar for 6 h. A solution of 1 M HCl was added to neutralize the solution. The aqueous fraction was extracted three times with ethyl acetate. The combined extracts were washed with brine (10 mL), dried over Na_2SO_4, and evaporated in vacuo, and the residue was purified by flash chromatography eluting with hexane/EtOAc (1:2) and recrystallized from 95% EtOH to give **7** (23 mg, 83%) as a white solid: ^{1}H NMR ($CDCl_3$): δ 1.37 (m, 4H), 1.62 (p, J = 7.2 Hz, 2H), 1.73 (t, J = 7.0 Hz, 2H), 2.35 (t, J = 7.2 Hz, 2H), 2.57 (t, J = 7.2 Hz, 2H), 3.34 (d, J = 7.2 Hz, 2H), 7.10 (t, J = 7.2 Hz, 1H), 7.32 (t, J = 8.0 Hz, 2H), 7.37 (s, 1H), 7.52 (d, J = 7.2 Hz, 2H); ^{13}C NMR ($CDCl_3$): δ 23.9, 25.5, 28.9, 29.1, 34.5, 37.8, 41.0, 119.9, 124.3, 129.1, 138.1, 171.4, 205.7. EI-MS (M^+): 279.2, 247.2, 232.0, 190.2, 135.1, 94.1. HRMS Calcd for $C_{15}H_{21}NO_2S$ 279.1288, found 279.1283. Anal. Calcd for $C_{15}H_{21}NO_2S$: C, 64.48; H, 7.58; N, 5.01; S, 11.47. Found: C, 64.39; H, 7.53; N, 4.77; S, 11.17.

4.8. 9-Acetylthio-8-oxo-*N*-phenylnonanamide (8)

To a solution of bromomethylketone **17** (326 mg, 1 mmol) in 5 mL DMF was added potassium thioacetate (240 mg, 2.1 mmol). The cloudy orange-yellow solution was stirred overnight at room temperature and then diluted with 10 mL of water. The aqueous fraction was extracted three times with ethyl acetate. The combined extracts were washed with brine (10 mL), dried over Na_2SO_4, and evaporated in vacuo, and the residue was purified by flash chromatography eluting with hexane/EtOAc (1:1) to give **8** (276 mg, 86%) as a white solid: ^{1}H NMR ($CDCl_3$): δ 1.36 (m, 4H), 1.62 (p, J = 7.0 Hz, 2H), 1.73 (t, J = 7.0 Hz, 2H), 2.34 (t, J = 7.0 Hz, 2H), 2.39 (s, 3H), 2.56 (t, J = 7.0 Hz, 2H), 3.74 (s, 2H), 7.09 (t, J = 7.0 Hz, 1H), 7.31 (t, J = 7.5 Hz, 2H), 7.43 (s, 1H), 7.52 (d, J = 7.5 Hz, 2H); ^{13}C NMR ($CDCl_3$): δ 23.7, 25.5, 28.8, 29.0, 30.4, 37.7, 39.3, 41.8, 120.0, 124.4, 129.2, 138.3, 171.6, 194.9, 204.3. EI-MS (M^+): 321.2, 279.2, 232.0, 135.1, 93.1. HRMS Calcd for $C_{17}H_{23}NO_3S$ 321.1393, found 321.1392. Anal. Calcd for $C_{17}H_{23}NO_3S$: C, 63.52; H, 7.21; N, 4.36; S, 9.98. Found: C, 63.46; H, 7.33; N, 4.37; S, 9.93.

4.9. 6-(2-Acetylthio[acetylamino])-*N*-phenylhexanamide (9)

To a stirred solution of (acetylthio)acetic acid (200 mg, 1.5 mmol) in anhydrous DMF (5 mL) were added PyBOP (781 mg, 1.5 mmol) and DIPEA (0.78 mL, 4.5 mmol) at room temperature. Then **22** hydrochloric acid salt (365 mg, 1.5 mmol) was added to the reaction mixture in one portion. The reaction mixture was stirred overnight. The solvent was removed under vacuum, and the residue was dissolved in ethyl acetate. The organic phase was washed with 0.5 N HCl solution, saturated $NaHCO_3$ and brine. The organic phase was then dried over Na_2SO_4, and evaporated in vacuo. The crude product was recrystallized from chloroform to give **9** (425 mg, 88%). ^{1}H NMR ($CDCl_3$): δ 1.35 (t, J = 7.0 Hz, 2H), 1.50 (t, J = 7.0 Hz, 2H), 1.72 (t, J = 7.0 Hz, 2H), 2.35 (t, J = 7.0 Hz, 2H), 2.37 (s, 3H), 3.21 (t, J = 6.5 Hz, 2H), 3.53 (s, 2H), 6.50 (br s, 1H), 7.07 (t, J = 7.0 Hz, 1H), 7.29 (t, J = 8.0 Hz, 2H), 7.56 (d, J = 8.0 Hz, 2H), 8.14 (s, 1H); ^{13}C NMR ($CDCl_3$): δ 26.4, 29.2, 30.4, 33.2, 37.4, 39.6, 46.5, 120.0, 124.2, 129.0, 138.4, 168.4, 171.7, 196.0. EI-MS (M^+): 322.4, 280.3, 188.3, 114.2, 93.2. HRMS Calcd for $C_{16}H_{22}N_2O_3S$ 322.1352, found 322.1350.

4.10. 9-Methylthio-8-oxo-*N*-phenylnonanamide (10)

To a solution of bromomethylketone **17** (32.6 mg, 0.1 mmol) in 2 mL DMF was added sodium thiomethoxide (7.7 mg, 0.11 mmol). The solution was stirred overnight at room temperature and then diluted with 10 mL of water. The aqueous fraction was extracted three times with ethyl acetate. The combined extracts were washed with brine (10 mL), dried over Na_2SO_4, and evaporated in vacuo, and the residue was purified by flash chromatography eluting with hexane/EtOAc (2:1) to give **10** (23.4 mg, 80%) as a white solid. ^{1}H NMR ($CDCl_3$): δ 1.38 (m, 4H), 1.63 (t, J = 6.0 Hz, 2H), 1.75 (d, J = 8.0 Hz, 2H), 2.07 (s, 3H), 2.36 (t, J = 7.2 Hz, 2H), 2.62 (t, J = 7.2 Hz, 2H), 3.17 (s, 2H), 7.10 (m, 1H), 7.25 (br s, 1H), 7.32 (t, J = 8.0 Hz, 2H), 7.52 (d, J = 8.0 Hz, 2H); ^{13}C NMR ($CDCl_3$): δ 15.9, 23.8, 25.5, 28.9, 29.0, 37.8, 40.2, 43.1, 119.9, 124.4, 129.2, 138.2, 171.4, 205.9. EI-MS (M^+): 293.2, 232.1, 200.1, 135.1, 93.1. HRMS Calcd for $C_{16}H_{23}NO_2S$ 293.1444, found 293.1443. Anal. Calcd for

$C_{16}H_{23}NO_2S$: C, 65.49; H, 7.90; N, 4.77; S, 10.93. Found: C, 65.27; H, 7.73; N, 4.67; S, 10.73.

4.11. 8,9-Dihydroxy-*N*-phenylnonanamide (11)

To a solution of **15** (1.0 g, 4.33 mmol) in *tert*-butanol (18 mL) and water (2 mL) at 0 °C were added *N*-methylmorpholine *N*-oxide (NMO) (608 mg, 5.20 mmol) and osmium tetroxide (33 mg, 0.13 mmol). The mixture was stirred at 0 °C for 1 h and then overnight at room temperature. Aqueous sodium thiosulfate was added, and the mixture was stirred at room temperature for another 0.5 h. The aqueous fraction was extracted three times with ethyl acetate. The combined extracts were washed with brine (10 mL), dried over Na_2SO_4, and evaporated in vacuo, and the residue was purified by flash chromatography eluting with hexane/EtOAc (1:1) to give **11** (884 mg, 77%) as a white solid: ^{1}H NMR ($CDCl_3$): δ 1.26 (m, 8H), 1.58 (m, 2H), 2.23 (t, J = 7.2 Hz, 2H), 3.27 (dd, J = 8.0 Hz, 11.2 Hz, 1H), 3.43 (dd, J = 4.8 Hz, 12.8 Hz, 1H), 3.50 (br d, J = 3.2 Hz, 1H), 6.96 (t, J = 7.2 Hz, 1H), 7.18 (t, J = 8.0 Hz, 1H), 7.42 (d, J = 8.0 Hz, 2H), 8.42 (m, 1H); EI-MS (M^+): 265.2, 234.2, 135.1, 93.1. HRMS Calcd for $C_{15}H_{23}NO_3$ 265.1672, found 265.1669. Anal. Calcd for $C_{15}H_{23}NO_3$: C, 67.90; H, 8.74; N, 5.28. Found: C, 67.92; H, 8.66; N, 5.26.

4.12. *N*-Phenyl-8-nonenamide oxide (12)

To a stirred solution of **15** (2.31 g, 10 mmol) in 100 mL of methylene chloride was added 77% *m*CPBA (3.36 g, 15 mmol) at 0 °C and then the reaction mixture was stirred overnight at 0 °C. The reaction was monitored by TLC and was quenched by the addition of a saturated $NaHCO_3$ solution. The mixture was extracted with ethyl acetate (3× 100 mL), and the combined organic extracts were washed with brine and dried over anhydrous Na_2SO_4. The solvent was removed under vacuum, and the residue was purified by flash chromatography eluting with hexane/EtOAc (3:1) to give **12** (2.10 g, 85%) as a white solid. ^{1}H NMR ($CDCl_3$): δ 1.33 (m, 4H), 1.43 (m, 2H), 1.49 (d, J = 4.8 Hz, 2H), 1.69 (br s, 2H), 2.33 (t, J = 7.2 Hz, 2H), 2.45 (dd, J = 2.4 Hz, 4.8 Hz, 1H), 2.73 (d, J = 4.4 Hz, 1H), 2.89 (br s, 1H), 7.06 (t, J = 6.8 Hz, 1H), 7.26 (t, J = 7.2 Hz, 1H), 7.55 (d, J = 8.0 Hz, 2H), 8.42 (m, 1H); ^{13}C NMR ($CDCl_3$): δ 25.6, 25.9, 28.5, 29.2, 32.4, 37.4, 47.1, 52.5, 120.0, 123.9, 128.7, 138.3, 172.0. EI-MS (M^+): 247.2, 135.1, 93.1. HRMS Calcd for $C_{15}H_{21}NO_2$ 247.1567, found 247.1566. Anal. Calcd for $C_{15}H_{21}NO_2$: C, 72.84; H, 8.56; N, 5.66. Found: C, 72.77; H, 8.62; N, 5.62.

4.13. *N*-Phenyl-8-nonenamide (15)

To a solution of the 8-nonenoic acid[19] (**14**, 3.2 g, 0.02 mol) in dry DMF (100 mL) was carefully added DIC (3.41 mL, 0.022 mol) at 0 °C followed by HOBt (2.97 g, 0.022 mol) and DIPEA (11.5 mL, 0.06 mol). After being stirred for 10 min a solution of aniline (2.01 mL, 0.022 mol) in 10 mL DMF was added dropwise to the reaction mixture at 0 °C. Stirring was continued for 1 h at 0 °C and then overnight at room temperature. Most of the DMF was evaporated with a high vacuum rotary evaporator, and the residue was dissolved to 500 mL of ethyl acetate. The organic phase was washed with saturated NH_4Cl solution (3× 50 mL) followed by brine (3× 50 mL) and dried over anhydrous Na_2SO_4. The solvent was removed under vacuum, and the residue was purified by flash chromatography eluting with hexane/EtOAc (5:1) to give **15** (4.27 g, 92%) as a white solid. ^{1}H NMR ($CDCl_3$): δ 1.28 (m, 6H), 1.65 (t, J = 7.0 Hz, 2H), 1.98 (t, J = 7.0 Hz, 2H), 2.34 (t, J = 7.5 Hz, 2H), 4.90-4.98 (2H), 5.74 (m, 1H), 7.00 (t, J = 7.5 Hz, 1H), 7.61 (d, J = 8.0 Hz, 2H), 9.41 (s, 1H); ^{13}C NMR ($CDCl_3$): δ 25.6, 28.5, 28.6, 28.9, 33.5, 37.1, 114.0, 120.2, 123.7, 128.3, 138.2, 138.3, 172.6. CI-MS (M^++1): 232.1, 139.1, 93.1. HRMS Calcd for $C_{15}H_{22}NO$ 232.1696, found 232.1695. Anal. Calcd for $C_{15}H_{21}NO$: C, 77.88; H, 9.15; N, 6.05. Found: C, 77.59; H, 9.27; N, 5.92.

4.14. 9-Bromo-8-hydroxy-*N*-phenylnonanamide (16)

A solution of **12** (1.0 g, 4.0 mmol) in peroxide-free THF (5 mL) was added dropwise to a stirred mixture of AcOH/THF/saturated aq KBr (2/1/1, 40 mL) at 0 °C under argon. After 10 h, the reaction mixture was reduced in vacuo to one-quarter volume, diluted with H_2O (50 mL), and extracted three times with Et_2O. The combined ethereal extracts were washed with 10% aqueous $NaHCO_3$ solution, H_2O, brine, dried over Na_2SO_4, and evaporated to dryness in vacuo, and the residue was used for the next step directly without further purification.

4.15. 9-Bromo-8-oxo-*N*-phenylnonanamide (17)

The above mixture of bromohydrins **16** (900 mg, 2.74 mmol) in diethyl ether (4 mL) was added dropwise to a 0 °C solution of 30 mL of Jones reagent in 15 mL of diethyl ether. After 5 h, the dark green mixture was diluted with 50 mL of water, and the aqueous fraction was extracted three times with Et_2O. The combined ethereal extracts were washed with brine (10 mL), dried over Na_2SO_4, and evaporated in vacuo, and the residue was purified by flash chromatography eluting with hexane/EtOAc (2:1) to give **17** (607 mg, 68%) as a white solid: ^{1}H NMR ($CDCl_3$): δ 1.36 (m, 4H), 1.62 (p, J = 7.0 Hz, 2H), 1.73 (p, J = 7.0 Hz, 2H), 2.35 (t, J = 7.5 Hz, 2H), 2.65 (t, J = 7.5 Hz, 2H), 3.88 (s, 2H), 7.10 (t, J = 7.5 Hz, 1H), 7.31 (t, J = 8.0 Hz, 2H), 7.52 (d, J = 7.0 Hz, 2H); ^{13}C NMR ($CDCl_3$): δ 23.7, 25.5, 28.8, 29.0, 34.5, 37.4, 39.9, 120.0, 124.4, 129.2, 138.1, 171.4, 202.4. EI-MS (M^+): 327.1, 232.0, 135.1, 93.1. HRMS Calcd for $C_{15}H_{20}NO_2Br$ 325.0672 and 327.0651, found 325.0674 and 327.0649.

4.16. 9-(*tert*-Butyldimethylsilanyloxy)-8-hydroxy-*N*-phenylnonanamide (18)

To a solution of **11** (265 mg, 1.0 mmol) in anhydrous DMF (2 mL) at 0 °C were added imidazole (134 mg, 2.0 mmol) and then *tert*-butyldimethylsilyl chloride (165 mg, 1.1 mmol). The mixture was stirred overnight at room temperature and then aqueous sodium thiosulfate was added, and the mixture was stirred at room

temperature for another 0.5 h. The aqueous fraction was extracted three times with ethyl acetate. The combined extracts were washed with brine (10 mL), dried over Na_2SO_4, and evaporated in vacuo, and the residue was purified by flash chromatography eluting with hexane/EtOAc (1:1) to give **11** (884 mg, 77%) as a white solid. ^{1}H NMR ($CDCl_3$): δ 0.08 (s, 6H), 0.91 (s, 9H), 1.36 (m, 8H), 1.72 (m, 2H), 2.34 (t, J = 7.2 Hz, 2H), 3.38 (t, J = 8.4 Hz, 1H), 3.62 (d, J = 10.8 Hz, 2H), 7.09 (t, J = 7.2 Hz, 1H), 7.31 (t, J = 8.0 Hz, 2H), 7.47 (s, 1H), 7.53 (d, J = 7.6 Hz, 2H); ^{13}C NMR ($CDCl_3$): δ −5.3, 18.5, 25.7, 25.8, 26.1, 26.2, 29.3, 29.6, 32.9, 37.9, 67.4, 72.0, 119.9, 124.3, 129.1, 138.2, 171.6. EI-MS: 322.4, 306.4, 230.5, 93.2. HRMS Calcd for $C_{21}H_{38}NO_3Si$ 380.2615, found 380.2612.

4.17. 9-(*tert*-Butyldimethylsilanyloxy)-8-oxo-*N*-phenyl-nonanamide (19)

A mixture of 1.0 g of $KMnO_4$ and 0.5 g of $CuSO_4{\cdot}5H_2O$ was ground to a fine powder and added to **18** (38 mg, 0.1 mmol) in 3 mL of benzene at room temperature. The reaction mixture was then heated to 70 °C for two days. The solid was filtered and washed with benzene. The combined filtration was evaporated in vacuo, and the residue was purified by flash chromatography eluting with hexane/EtOAc (1:1) to give **19** (20 mg, 52%). ^{1}H NMR ($CDCl_3$): δ 0.10 (s, 6H), 0.93 (s, 9H), 1.38 (br s, 4H), 1.60 (m, 2H), 1.75 (t, J = 7.2 Hz, 2H), 2.35 (t, J = 7.6 Hz, 2H), 2.50 (t, J = 7.6 Hz, 2H), 4.17 (s, 2H), 7.11 (t, J = 7.6 Hz, 1H), 7.22 (br s, 1H), 7.32 (t, J = 8.0 Hz, 2H), 7.43 (s, 1H), 7.53 (d, J = 7.6 Hz, 2H); ^{13}C NMR ($CDCl_3$): δ −5.3, 18.5, 23.2, 25.5, 26.0, 29.0, 29.1, 37.8, 38.4, 69.5, 119.9, 124.4, 129.2, 138.2, 171.5, 211.4. HRMS Calcd for $C_{21}H_{36}NO_3Si$ 378.2459, found 378.2468.

4.18. 6-(*tert*-Butyloxycarbonylamino)-*N*-phenylhexanamide (21)

To a solution of the 6-(*tert*-butoxycarbonylamino)hexanoic acid (**20**) (4.6 g, 0.02 mol) in dry DMF (200 mL) was carefully added DIC (3.41 mL, 0.022 mol) at 0 °C followed by HOBt (2.97 g, 0.022 mol) and DIPEA (11.5 mL, 0.06 mol). After being stirred for 10 min, a solution of aniline (2.01 mL, 0.022 mol) in 10 mL DMF was added dropwise to the reaction mixture at 0 °C. Stirring was continued for 1 h at 0 °C and then overnight at room temperature. Most of the DMF was evaporated with a high vacuum rotary evaporator, and the residue was dissolved in 500 mL of ethyl acetate. The organic phase was washed with saturated NH_4Cl solution (3× 50 mL) followed by brine (3× 50 mL) and dried over anhydrous Na_2SO_4. The solvent was removed under vacuum, and the residue was purified by flash chromatography eluting with hexane/EtOAc (5:1) to give **21** (5.63 g, 92%) as a white solid. ^{1}H NMR ($CDCl_3$): δ 1.38 (t, J = 7.0 Hz, 2H), 1.44 (s, 9H), 1.50 (t, J = 7.0 Hz, 2H), 1.73 (t, J = 7.5 Hz, 2H), 2.35 (t, J = 7.0 Hz, 2H), 3.11 (d, J = 6.0 Hz, 2H), 4.65 (br s, 1H), 7.08 (t, J = 7.0 Hz, 1H), 7.30 (t, J = 7.5 Hz, 2H), 7.54 (d, J = 7.5 Hz, 2H), 7.76 (s, 1H); ^{13}C NMR ($CDCl_3$): 23.7, 25.3, 26.5, 28.6, 29.9, 37.6, 40.7, 120.0, 124.3, 129.1, 138.3, 156.3, 171.6. EI-MS (M^+): 306.4, 250.3, 233.2, 187.4, 135.2, 93.2. HRMS Calcd for $C_{17}H_{26}N_2O_3$ 306.1938, found 306.1940.

4.19. 6-Amino-*N*-phenylhexanamide (22)

To a solution of **21** (3.06 g, 10 mmol) in 50 mL of methylene chloride was added 50 mL TFA in one portion at room temperature. The reaction mixture was stirred for another 1 h and then was evaporated under vacuum to remove the solvent. The crude **22** TFA salt was then dissolved in 10 mL of ethanol containing 0.5 mL of HCl acid. The mixture was evaporated under vacuum again to form **22** HCl salt (2.41 g, 99%), which was used to make **3** and **9** without further purification.

4.20. 7-Oxiranyl-heptanoic acid phenylamide (13)

To a stirred solution of **12** (100 mg, 0.40 mmol) in 2 mL of methanol was added thiourea (62 mg, 0.80 mmol) at room temperature then the reaction mixture was stirred overnight. The solvent was evaporated, the resulting residue partitioned between ethyl acetate (50 mL) and water (10 mL), and the organic phase washed with brine and dried over anhydrous Na_2SO_4. The solvent was removed under vacuum, and the residue was purified by flash chromatography eluting with hexane/EtOAc (5:1) to give **13** (92 mg, 88%) as a white solid: ^{1}H NMR ($CDCl_3$): δ 1.36 (m, 6H), 1.50 (m, 2H), 1.80 (m, 2H), 2.13 (d, J = 6.0 Hz, 1H), 2.34 (t, J = 7.2 Hz, 2H), 2.49 (d, J = 6.4 Hz, 1H), 2.85 (m, 1H), 7.05 (t, J = 6.4 Hz, 1H), 7.26 (t, J = 7.2 Hz, 2H), 7.57 (d, J = 8.0 Hz, 2H), 7.75 (s, 1H); ^{13}C NMR ($CDCl_3$): δ 25.6, 26.1, 29.1, 29.3, 36.2, 36.6, 37.7, 120.0, 124.3, 129.0, 138.2, 171.8. HRMS (ESI) calcd for $C_{15}H_{22}NOS$ 264.1422, found 264.1417. Anal. Calcd for $C_{15}H_{21}NOS$: C, 68.40; H, 8.04; N, 5.32; S, 12.17. Found: C, 68.14; H, 8.10; N, 5.21; S, 12.05.

4.21. HDACs and HDAC8 enzyme assays

The in vitro HDAC inhibition assay was performed using the HDAC fluorescent histone deacetylase activity assay kit (Biomol Research Laboratories, Plymouth Meeting, PA). A HeLa cell nuclear extract, which contains a number of HDAC isozymes and other nuclear factors, was used as the source of the HDAC activity. The final substrate concentration in the assay mixture was 50 μM. The reaction was allowed to proceed for 30 min at 37 °C before stopping the reaction. Test compounds were prepared as 1 mM stock solutions in DMSO (molecular biology grade, Sigma–Aldrich Co., St. Louis, MO) and stored at 0 °C. The final DMSO concentration per well was no more than 2%. Assays were performed in white polystyrene 96-well half-area assay plates (Corning, NY) and measured on an EM Fluorescence/Chemiluminescence Plate Reader (Molecular Devices Spectramax Gemini fluorometric detection device) with an excitation wavelength of 355 nm and an emission wavelength of 460 nm using SOFTmax PRO software.

The HDAC8 inhibition assay was performed using the HDAC8 fluorescent activity assay kit (Biomol Research

Laboratories, Plymouth Meeting, PA). The final substrate concentration in the assay mixture was 25 μM. The reaction was allowed to proceed for 45 min at 37 °C before stopping the reaction with the Fleur de Lys™ Developer II and then was incubated for 10 min at room temperature. The absorbance was determined as above for the mixture of HDACs. Test compounds were prepared as 1 mM stock solutions in DMSO (molecular biology grade, Sigma–Aldrich Co., St. Louis, MO) and stored at 0 °C. The final DMSO concentration per well was no more than 2%. Assays were performed as described above.

4.22. Procedure for detection of inhibitor hydrolysis during HDAC inhibition

Compound **8** was incubated with HeLa cell nuclear extract for 30 min at 37 °C before stopping the reaction with Fleur de Lys™ developer. The combined buffer solution (100 μL) was then extracted with ethyl acetate (1× 3 mL). The organic phase was combined and washed with brine and then dried over anhydrous Na_2SO_4. The solution was analyzed with a VG 70-250SE high-resolution mass spectrometer (ESI), resulting in a peak at 322.26 (M+1); the mass peak for the corresponding hydrolyzed compound (**7**) was not detected. The results indicate that **8** is not hydrolyzed during the inhibition of HDACs.

4.23. Cell culture, IFN and drug treatments, and transfection

Human 2fTGH cells were maintained in DMEM supplemented with 10% cosmic calf serum (HyClone). Transfection of 2fTGH cells was carried out using SuperFect reagent (Qiagen, Valencia, CA) according to the manufacturer's instructions. Treatment of cells with IFN was performed as indicated using 1000 units of IFN_α per mL. Trichostatin A (TSA, Upstate Biotechnology, Lake Placid, NY) was added at 400 ng/mL simultaneously with IFN_α. Sodium butyrate (Upstate Biotechnology) was added simultaneously with IFN_α at a final concentration of 5 mM. SAHA and its derivatives were also added simultaneously with IFN_α at a final concentration of 5 μM.

4.24. Plasmids and reporter gene assays

Luciferase assays were carried out according to the manufacturer's instructions (Promega) by co-transfecting the IFN-responsive 5xISRE luciferase reporter plasmid together with *Renilla* luciferase (Dual-Luciferase Reporter Assay) to normalize for transfection efficiency. The 5xISRE luciferase reporter gene contained five copies of the ISG54 ISRE element upstream of the TATA box and the firefly luciferase ORF. Cells were treated with IFN and HDAC inhibitors 24 h after transfection. Data represent means ± SD of triplicate samples.

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