

6-nitroBaP

Figure 1. 6-NitroBaP as a photoinducible NO-releasing agent.

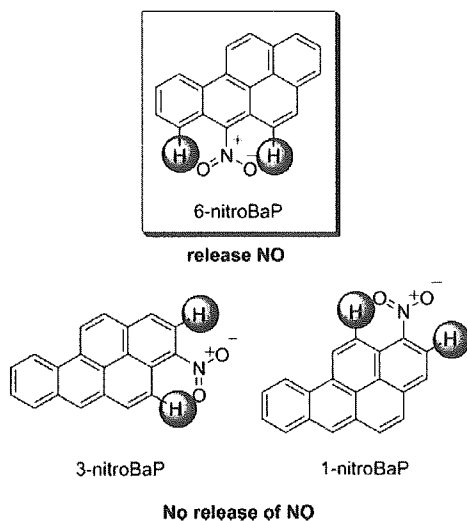


Figure 2. Structures of 1-, 3-, and 6-nitroBaP.

groups did not release NO (Figure 2) and the results of computational studies by our group¹⁰ as well as another,¹¹ it was suggested that the nonplanar torsional conformation of the nitro substituent in relation to the aromatic ring is important for the light-induced release of NO. In view of this finding, regulation of the nitro group conformation could give simpler nitrobenzene derivatives the ability to release NO. Contributing to this line of thought, we report that some nitrobenzene derivatives with extended π -electron conjugations have potent NO-releasing activity in response to visible-light irradiation. A simulation study and ultraviolet spectrum analysis of these compounds suggested that the NO-releasing activity was closely related to the conformation of the nitro group, the absorption wavelength, and the length of the conjugated π -electron system. Employing the photodependent cytotoxicity of compound **14** against HCT116 human colon cancer cells, it was demonstrated that nitrobenzene derivatives are useful as time- and site-controlled NO donors.

Results

Molecular Design. As NO donor candidates, we focused on 2,6-dimethylnitrobenzene derivatives, which are expected to have nonplanar conformations of the nitro group in their simple structures (Figure 3). Electron-donating groups (**2** and **3**) and olefins (**4–11**, and **14**) were chosen as substituents at the 4-position so that the derivatives would have maximum absorption at a longer wavelength, because it is desirable that compounds release NO under visible-light irradiation without affecting the living organisms. In addition, the extension of conjugated π -electron systems by the introduction of olefins at

the 4-position may increase the NO-releasing ability by stabilizing the oxyradical which is speculated to be formed concomitantly with the generation of NO.¹⁰ Compounds with one methyl group (**12**) and without a methyl group (**13**) were also prepared as reference compounds.

ESR Analysis. Detection of NO released from nitrobenzene derivatives was carried out by an ESR spin-trapping method with *N*-methyl-D-glucamine dithiocarbamate (MGD)–Fe²⁺ complex, which reacts with NO to give a [(MGD)₂–Fe²⁺–NO] stable paramagnetic complex.¹² On ESR spectroscopy, the complex is observed as a broadened three-line spectrum consisting of $a^N = 1.25$ mT and $g^{\text{dso}} = 2.04$. The prepared 2, 6-dimethylnitrobenzene derivatives were photoirradiated in aqueous DMSO and subsequently subjected to ESR spectroscopy.

No ESR signal was detected with compounds **1**, **2**, and **3** bearing an OH and the NMe₂ group at the 4-position of 2,6-dimethylnitrobenzene, respectively (data not shown), whereas a 5-min photoirradiation¹³ of compound **4** bearing a styrenyl group at the 4-position exhibited a characteristic three-line spectrum on ESR (Figure 4A), which indicates that NO was released from compound **4** by photoirradiation. NO-releasing activity was distinctly dependent on the two methyl groups introduced at the ortho position to the nitro group on the phenyl ring. The lack of the methyl groups (**12** and **13**) led to a much less potent NO donor and a compound devoid of the ability to release NO, respectively (Figure 4). We next evaluated the ESR signal intensity of compound **4** and its derivatives **5–11** after 1-, 3-, and 5-min photoirradiation (Figure 5). All of the tested compounds were shown to release NO during these photoirradiation periods. Time-dependent increases in the ESR signal intensity indicated the photolytic generation of NO from these compounds. With the extension of the conjugated π -electron system (see **5–9**) the NO-releasing activity increased except for that of compound **7**, which was less potent than **4**. Compounds with longer conjugated π -electron systems showed greater activity when comparing **4** or **8** with **9**. Next, we studied the effect of substituents at the other phenyl ring of compound **4**. It was shown that 3',5'-dimethoxy compound **11** was more potent, while 2',6'-dimethyl analogue **10** significantly reduced the activity.

Griess Assay. For further confirmation of NO production via the photolysis of nitrobenzene derivatives, the Griess method¹⁴ was used to evaluate production by detecting NO₂⁻ resulting from NO autoxidation in aqueous solution. The visible absorption at 546 nm of the red color formed upon diazo coupling of the Griess reagents allowed for evaluation of the NO formed after photolysis of the nitrobenzene derivatives. After photoirradiation for 1 and 2 h, the absorbance at 546 nm was measured.

The Griess assay was used to investigate the NO-releasing efficiency of **4**, **5**, **8**, and **9**, positive compounds in the ESR assay, and **12** and **13**, negative compounds in the ESR assay (Figure 6). Compounds **4**, **5**, **8**, and **9** bearing two methyl groups at the ortho positions of the nitro group released NO in a time-

(11) Glenewinkel-Meyer, T.; Crim, F. F. *J. Mol. Struct.* **1995**, *337*, 209–224.

(12) (a) Komarov, A.; Mattson, D.; Jones, M. M.; Singh, P. K.; Lai, C. S. *Biochem. Biophys. Res. Commun.* **1993**, *195*, 1191–1198. (b) Pieper, M. G.; Lai, C. S. *Biochem. Biophys. Res. Commun.* **1996**, *219*, 584–590.

(13) Compounds prepared for this study were irradiated using a 300-W photoreactor lamp that produces light having a wavelength range of >300 nm.

(14) Green, L. C.; Wagner, D. A.; Glogowski, J.; Skipper, P. L.; Wishnok, J. S.; Tannenbaum, S. T. *Anal. Chem.* **1982**, *54*, 131–138.

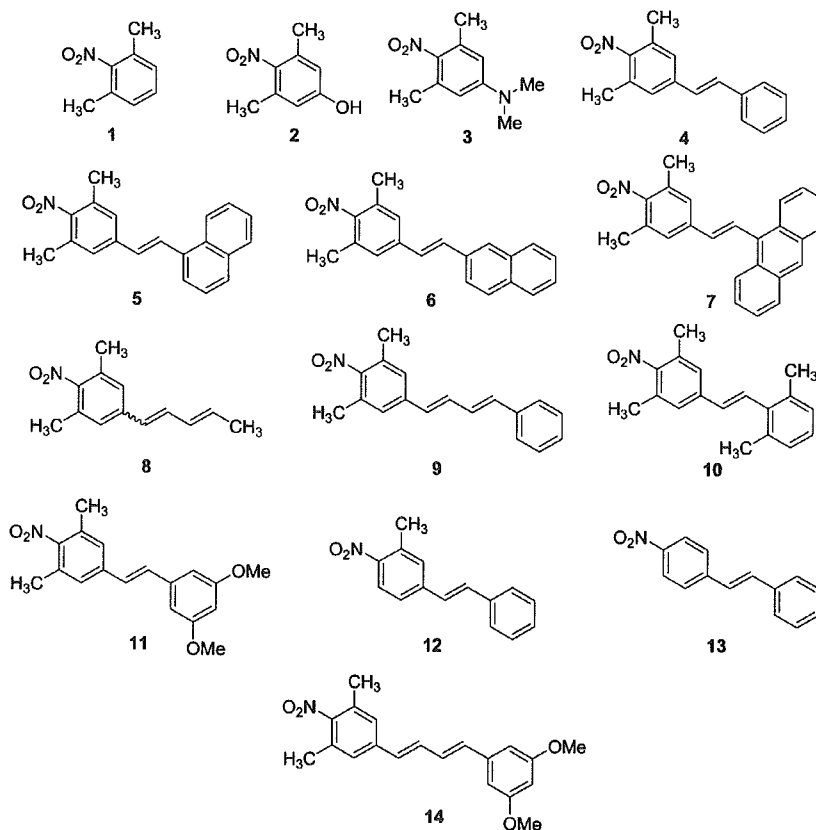


Figure 3. Structures of nitrobenzene derivatives.

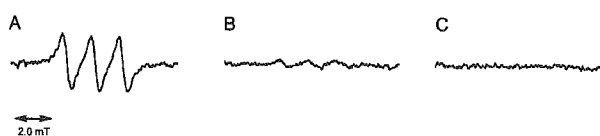


Figure 4. ESR spectra of $[(\text{MGD})_2\text{-Fe-NO}]$ complex after photoirradiation in the presence of compounds 4, 12, and 13. Samples contained 1 mM 4 (A), 12 (B), or 13 (C), 75 mM MGD, and 20 mM FeSO_4 in distilled water (containing 25% DMSO); ESR spectra were recorded after photoirradiation for 5 min with a modulation amplitude of 2.0 G and a microwave power of 16 mW.

dependent manner under visible light, while an extreme reduction in the NO-releasing activity was observed with compounds having a single methyl group (12) and lacking a methyl group (13). On comparing the four positive compounds (4, 5, 8, and 9), the longer their conjugated π -electron systems, the better NO donors they were. The results from the Griess assay were consistent with those obtained by ESR analysis.

To determine the chemical yield of the NO-releasing reaction, compound 9, the most active compound in both the ESR and Griess assays was subjected to a longer period of photoirradiation and the NO-release rate was measured by the Griess method (Figure 7). The generation of NO from compound 9 increased linearly until 6 h of photoirradiation and then reached a plateau. The yield of NO formation for the photochemical reaction determined by the Griess method was 55%.

Cytotoxicity Against Cancer Cells. It has been reported that NO is a mediator of the cytotoxic action of macrophages toward tumor cells through inhibition of mitochondrial enzyme activity and DNA synthesis.¹⁵ Therefore, we determined whether NO

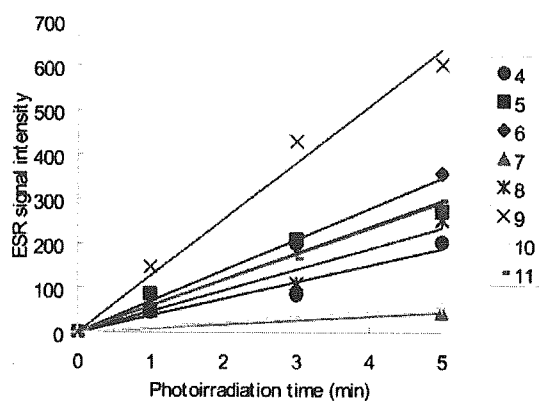


Figure 5. ESR signal intensities of $[(\text{MGD})_2\text{-Fe-NO}]$ complex after photoirradiation in the presence of compounds 4–11. Samples contained 1 mM 4–11, 75 mM MGD, and 20 mM FeSO_4 in water (containing 25% DMSO); ESR spectra were recorded after photoirradiation for 1, 3, or 5 min with a modulation amplitude of 2.0 G and a microwave power of 16 mW.

induced from nitrobenzene derivatives by photoirradiation can function as a cytotoxic agent. For the application of our NO donors in a biological study, we prepared compound 14. In the ESR and the Griess assays, the NO-releasing activity of compound 14 was comparable to that of compound 9 (see Supporting Information), the most potent compound among all those prepared for this study. Compound 14 has the advantage of solubility in aqueous media compared with compound 9. It

(15) Stuehr, D. J.; Gross, S. S.; Sakuma, I.; Levi, R.; Nathan, C. F. *J. Expl. Med.* 1989, 169, 1011–1020.

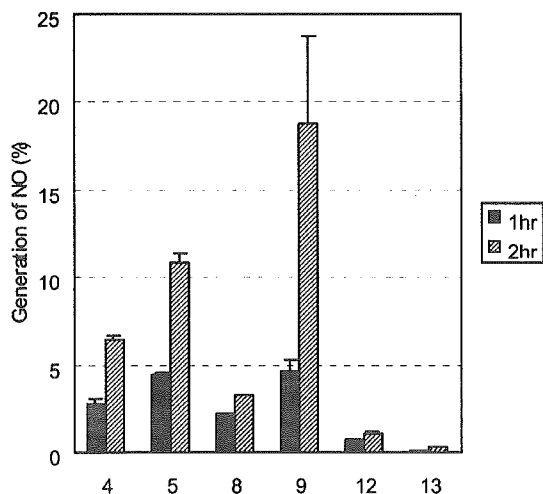


Figure 6. Griess assay results for compounds 4, 5, 8, 9, 12, and 13 ($n = 3$, mean \pm SD).

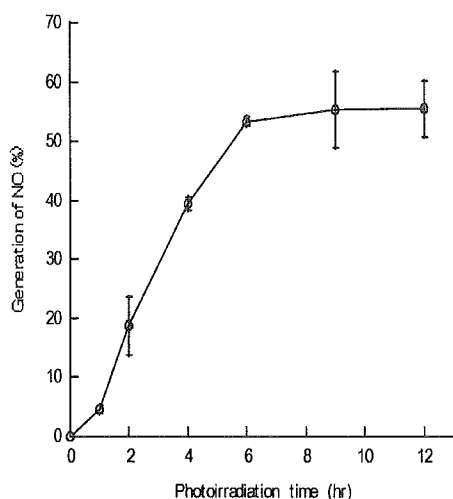


Figure 7. NO-release rate of compound 9 ($n = 3$, mean \pm SD).

was more easily available for the cellular experiments as a DMSO solution. In the presence of a $2.5 \mu\text{M}$ concentration of compound 14, light of 330–380 nm wavelength was shed for 1 min on a fixed area of a culture plate containing human colon cancer HCT116 cells.¹⁶ The cells were observed after a 24-h incubation at 37°C under dark conditions. As shown in Figure 8, the cells had detached and appeared dead within the area of irradiation (Figure 8a), which indicates that the cytotoxicity was quite dependent on the photoirradiation. Treatment with compound 14 without photoirradiation had no effect on the cells in 24 h. The photoirradiation alone without compound 14 also had no effect. This cytotoxic effect was diminished in the presence of NADH and P450 nor, an NO reductase (Figure 8c), which confirmed that NO was mainly responsible for the cytotoxicity against the HCT 116 cells. There were no changes at a position 3.5 mm from the center of irradiation, i.e., outside the photoirradiation area, regardless of the absence (Figure 8b) or the presence of P450 nor (Figure 8d). Preirradiation to the solution of compound 14 for 30 min abolished its cytotoxic effects,

(16) NO release from compound 14 under assay conditions was confirmed by the Griess Method (see Supporting Information).

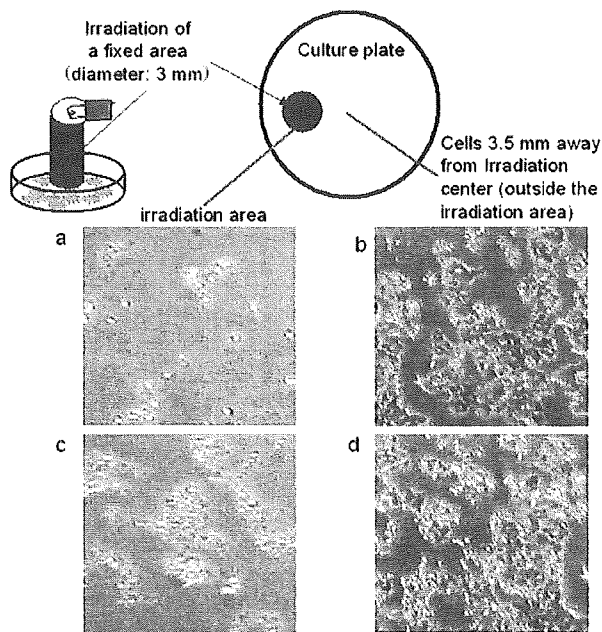


Figure 8. Cytotoxic activity of NO released from compound 14 under photoirradiation. Representative phase-contrast images of cells within the irradiation area in the absence (a) and the presence (c) of P450 nor are shown. Cells at 3.5 mm away from the irradiation center in the absence (b) and the presence (d) of P450 nor are also presented. The experiments were duplicated, and at least 4 areas were observed in each culture plate. In all experiments, the same results were obtained.

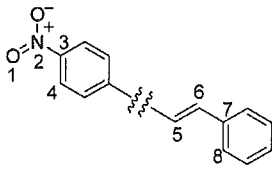
indicating that the stable products after liberation of NO or photoinduced decomposition are nontoxic.

Discussion

MM2 calculations and the ultraviolet–visible-light absorption spectra measurements were carried out to determine which characteristic is necessary for NO generation under visible-light irradiation (Table 1).

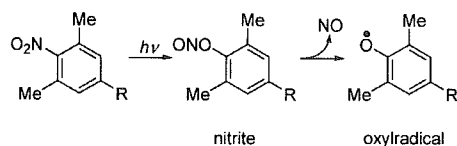
It is assumed that a nitroarene gives rise to NO via nitritearene, which is produced from the nitroarene by an intramolecular rearrangement mechanism (Figure 9), and the nonplanar torsional conformation of the nitro group in relation to the aromatic ring gives an advantage in the nitro-to-nitrite rearrangement.^{10,11} Therefore, the dihedral angle between the plane of the nitro group and the phenyl ring ($\Delta^{\circ}\text{N}^2\text{C}^3\text{C}^4$) for compounds 1–13 was initially calculated to clarify the effect of the two methyl groups on the conformation of the nitro group. The dihedral angles for dimethyl compounds 1–11 were around 45° , while those of monomethyl 12 and no-methyl 13 were 0° . Considering this calculation result and the fact that compounds 12 and 13 did not generate NO efficiently, it was suggested that two methyl groups at the ortho positions of the nitro group are indispensable for inclining the nitro group to the plane of the phenyl ring and for the subsequent intramolecular rearrangement from nitro to nitrite.

Another important factor is the intensity of light energy absorption, which is considered to be essential for the excitation of compounds or further torsional conformation change of the nitro group.¹¹ Indeed, compounds with a longer conjugated π -electron system, which were expected to have maximum absorption at a longer wavelength, were inclined to enhance

Table 1. Results of MM2 Calculations and Ultraviolet Absorption Measurements


comps	O ⁻ N ⁺ C ³ C ⁴ ^a (deg)	C ⁵ C ⁶ C ⁷ C ⁸ ^b (deg)	λ max (nm)	ϵ max ($\times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$)	ESR signal ^c ($\times 10^2$ au)
1	44.8		255	0.14	nd
2	43.6		354	0.14	nd
3	44.4		396	0.65	nd
4	44.6	30.3	307	2.4	2.0
5	44.6	50.3	329	2.0	2.8
6	44.7	30.3	320	2.7	3.6
7	44.6	79.8	389	1.1	0.4
8	44.6		289	1.4	2.5
9	44.6	28.5	332	4.3	6.0
10	44.7	66.7	287	1.6	0.6
11	44.7	32.1	308	2.6	3.0
12	0.1	30.0	343	2.2	0.4
13	0.0	30.0	349	2.6	nd

^a Dihedral angle (deg) between the planes of the nitro group and the phenyl moiety. ^b Dihedral angle (deg) between the ethylene moiety and the aromatic ring. ^c ESR signal intensity of [(MGD)₂-Fe-NO] complex after 5-min irradiation. nd = not detected.

**Figure 9.** Predicted mechanism for NO generation from 4-substituted-2,6-dimethylnitrobenzene derivatives.

the NO-releasing activity. The results of the ultraviolet–visible-light spectroscopic investigation of nitrobenzene derivatives were in good agreement with those obtained by the ESR assay. Specifically, all of the compounds with an adequate ability to provide NO (4, 5, 6, 8, 9, and 11) had strong absorption in the range of irradiating light (wavelength of >300 nm), while compounds having weak absorption (1 and 3) under these conditions did not generate NO although they had two methyl groups at the ortho positions of the nitro group. These results support the idea that NO is released from nitrobenzene derivatives by light energy absorption and not by any other factor.

However, compounds 7 and 10, which have two methyl groups, had adequate absorption at wavelengths of >300 nm and seemed to have long conjugated π -electron systems, hardly having any ability to release NO. The calculated dihedral angle between the ethylene moiety and the aromatic ring ($\Delta C^5C^6C^7C^8$) of compounds 7 and 10 was 79.8° and 66.7°, respectively. These values were greater than those of other compounds. This suggested that compounds 7 and 10 have difficulty in creating a planar structure, and therefore there is little conjugation between the two aromatic rings. It can be concluded that the lack of a conjugated π -electron system made it difficult for compounds 7 and 10 to absorb the light energy necessary for generating NO.¹⁷

(17) In the case of compound 7, the absorption at 386 nm, which is due to the anthracene ring, has little to do with NO generation since the nitrobenzene moiety and the anthracene ring are not conjugated.

Longer conjugated π -electron systems can also contribute to the resonance stabilization of the oxyradicals generated by N–O bond fission of the nitrite. The destabilization of the oxyradical caused by the lack of a π -conjugated system may be the reason that compounds 3, 7, and 10 did not have a significant ability to release NO.

Unfortunately, the oxyradical, which is considered to be formed concomitantly with the generation of NO, could not be detected because of its instability. However, the result of the cellular assay using compound 14 indicated that the degradation products derived from the oxyradical did not appear to interfere with the biological NO experiment. Therefore, nitrobenzene derivatives may be useful NO donors, with the advantage that the time of light exposure, the duration of NO release, and the site of NO action can be controlled.

All the synthesized compounds were very stable in crystal forms under a dark condition. It was also confirmed that the photoinduced cytotoxic effect of compound 14 was maintained at least for 4 weeks when stored at -20 °C as a concentrated solution in DMSO. This stability of the compounds in storage appears to give usefulness in the biological applications as NO-releasing reagents.

Conclusion

In the present study, we characterized several 4-substituted-2,6-dimethylnitrobenzenes as a new type of NO donor. The computational study and ultraviolet–visible-light spectrum analysis also highlighted the significance of the conformation of the nitro group, the intensity of light energy absorption, and the length of the conjugated π -electron system. More importantly, one of our compounds demonstrated its activity as an NO donor in a biological study.

Experimental Section

General Methods. Melting points were determined using a Yanagimoto micromelting point apparatus or a Büchi 545 melting point apparatus and were left uncorrected. Proton nuclear magnetic resonance spectra (¹H NMR) were recorded on a JEOL JNM-LA400 spectrometer with CDCl₃ as the solvent. Chemical shifts (δ) were reported in parts per million relative to the internal standard tetramethylsilane. Elemental analysis was performed with a Yanaco CHN CORDER NT-5 analyzer, and all values were within $\pm 0.4\%$ of the calculated values. High-resolution mass spectra (HRMS) were recorded on a JEOL JMS-SX102A mass spectrometer. GC–MS analyses were performed on a Shimadzu GCMS-QP2010. Ultraviolet–visible-light spectra were recorded on a HITACHI U-3000 spectrophotometer or an Agilent 8453 spectrophotometer. Compound 1 was purchased from Wako Pure Chemical Industries, and MGD was obtained from DOJINDO. All other reagents and solvents were purchased from Aldrich, Tokyo Kasei Kogyo, Wako Pure Chemical Industries, and Kanto Kagaku and used without purification. Flash column chromatography was performed using Silica Gel 60 (particle size 0.046–0.063 mm) supplied by Merck. In ESR analysis and the Griess assay, irradiation was performed through a Pyrex filter with a 300-W photoreflexor lamp.

3,5-Dimethyl-4-nitrophenol (2). Compound 2 was prepared by a previously reported method:¹⁸ mp 109–111 °C; ¹H NMR (CDCl₃, 400 MHz, δ ; ppm) 6.56 (2H, d, $J = 0.49$ Hz), 5.19 (1H, s), 2.30 (6H, s). MS (EI) m/z : 167 (M^+). Anal. Calcd for C₈H₉NO₃: C, 57.48; H, 5.43; N, 8.38. Found: C, 57.33; H, 5.55; N, 8.61.

(18) Gaude, D.; Le Goaller, R.; Pierre, J. L. *Synth. Commun.* **1986**, *16*, 63–68.

(3,5-Dimethyl-4-nitrophenyl)dimethylamine (3). Compound **3** was prepared by a previously reported method:¹⁹ mp 115–116 °C; ¹H NMR (CDCl₃, 400 MHz, δ; ppm) 6.31 (3H, s), 3.01 (6H, s), 2.37 (6H, s). MS (EI) *m/z*: 194 (M⁺). Anal. Calcd for C₁₀H₁₄N₂O₂: C, 61.84; H, 7.27; N, 14.42. Found: C, 61.71; H, 7.30; N, 14.58.

1,3-Dimethyl-2-nitro-5-[(1E,3E)-4-(3,5-dimethoxyphenyl)-1,3-butadienyl]benzene (14). **Step 1: Preparation of 3,5-Dimethyl-4-nitrobenzylbromide.** To phosphorus tribromide (1.16 g, 4.29 mmol) was added 3,5-dimethyl-4-nitrobenzyl alcohol²⁰ (489 mg, 2.70 mmol) under argon with cooling by an ice bath, and the solution was stirred for 5 h at room temperature. The reaction mixture was poured into water and extracted with CHCl₃. The CHCl₃ layer was separated, washed with brine, and dried over Na₂SO₄. Filtration and concentration in vacuo and purification by silica gel flash chromatography (*n*-hexane/AcOEt = 9/1) gave 610 mg (93%) of 3,5-dimethyl-4-nitrobenzylbromide as a pale yellow solid: mp 49–50 °C; ¹H NMR (CDCl₃, 400 MHz, δ; ppm) 7.15 (2H, s), 4.40 (2H, s), 2.31 (6H, s). MS (EI) *m/z*: 243 (M⁺, ⁷⁹Br), 245 (M⁺, ⁸¹Br).

Step 2: Preparation of Diethyl 3,5-Dimethyl-4-nitrobenzylphosphonate. A mixture of 3,5-dimethyl-4-nitrobenzylbromide (694 mg, 2.84 mmol) obtained above, tetrabutylammonium iodide (77 mg, 0.21 mmol), and triethyl phosphite (596 mg, 3.59 mmol) was stirred for 6 h at 120 °C. The reaction mixture was subjected to silica gel flash chromatography (CHCl₃/MeOH = 100/1) to give 873 mg (q.y.) of 3,5-dimethyl-4-nitrobenzylphosphonate as a pale yellow oil: ¹H NMR (CDCl₃, 400 MHz, δ; ppm) 7.06 (2H, d, *J* = 2.4 Hz), 4.10–4.02 (4H, m), 3.09 (2H, d, *J* = 22.2 Hz), 2.30 (6H, s), 1.28 (6H, t, *J* = 7.1 Hz). MS (EI) *m/z*: 301 (M⁺). HRMS: calcd for C₁₃H₂₀NO₃P, 301.108; found, 301.108.

Step 1': Preparation of 3-(3,5-Dimethoxyphenyl)acrylonitrile. To a suspension of sodium hydride (60%, 1.27 g, 31.8 mmol) in THF (30 mL) was added a solution of cyanomethylphosphonic acid diethyl ester (500 mg, 2.82 mmol) in THF (10 mL) under argon with cooling by an ice bath, and the solution was stirred for 15 min at room temperature. To the mixture was added to 3,5-dimethoxybenzaldehyde (656 mg, 3.95 mmol), and the reaction mixture was stirred for 17 h at room temperature. The mixture was poured into water and extracted with CHCl₃. The CHCl₃ layer was separated, washed with brine, and dried over Na₂SO₄. Filtration and concentration in vacuo and purification by silica gel flash chromatography (*n*-hexane/AcOEt = 4/1) gave 833 mg (q.y.) of 3-(3,5-dimethoxyphenyl)acrylonitrile as a white solid; ¹H NMR (CDCl₃, 400 MHz, δ; ppm) 7.32 (1H, d, *J* = 16.5 Hz), 6.57 (2H, d, *J* = 2.2 Hz), 6.53 (1H, t, *J* = 2.2 Hz), 5.85 (1H, d, *J* = 16.6 Hz), 3.81 (6H, s).

Step 2': Preparation of 3-(3,5-Dimethoxyphenyl)propenal. To a solution of 3-(3,5-dimethoxyphenyl)acrylonitrile (1.31 g, 7.56 mmol) obtained above in toluene (30 mL) was added diisobutyl aluminum hydride (1.0 M, 15.1 mL, 15.1 mmol) under argon at –50 °C, and the solution was stirred for 22 h at room temperature. The mixture was poured into 1 N aqueous HCl and extracted with AcOEt. The AcOEt layer was separated, washed with brine, and dried over Na₂SO₄. Filtration and concentration in vacuo and purification by silica gel flash chromatography (*n*-hexane/AcOEt = 4/1) gave 378 mg (26%) of 3-(3,5-dimethoxyphenyl)propenal as a pale yellow crystal; ¹H NMR (CDCl₃, 400 MHz, δ; ppm) 9.70 (1H, d, *J* = 10.9 Hz), 7.41 (1H, d, *J* = 15.9 Hz), 6.71–6.65 (3H, m), 6.55 (1H, t, *J* = 2.3 Hz), 3.83 (6H, s).

Step 3: Preparation of 1,3-Dimethyl-2-nitro-5-[(1E,3E)-4-(3,5-dimethoxyphenyl)-1,3-butadienyl]benzene (14). Compound **14** was prepared from diethyl 3,5-dimethyl-4-nitrobenzylphosphonate obtained in step 2 and 3-(3,5-dimethoxyphenyl)propenal obtained above using the same procedure described in step 1' in a 55% yield: mp 132–133 °C; ¹H NMR (CDCl₃, 400 MHz, δ; ppm) 7.16 (2H, s), 6.93 (2H, m),

6.62 (4H, m), 6.40 (1H, s), 3.83 (6H, s), 2.33 (6H, s). MS (EI) *m/z*: 339 (M⁺). Anal. Calcd for C₁₉H₁₉NO₃: C, 73.77; H, 6.19; N, 4.53. Found: C, 73.63; H, 6.23; N, 4.55.

Compounds **4–13** were prepared from the corresponding diethylphosphonates and aldehydes using the procedure described for **14** (step 1').

(E)-3,5-Dimethyl-4-nitrostilbene (4): mp 101–103 °C; ¹H NMR (CDCl₃, 400 MHz, δ; ppm) 7.52 (2H, d, *J* = 7.3 Hz), 7.38 (2H, dd, *J* = 7.1, 7.8 Hz), 7.32–7.28 (1H, m), 7.25 (2H, s), 7.14 (1H, d, *J* = 16.3 Hz), 7.02 (1H, d, *J* = 16.3 Hz), 2.35 (6H, s). MS (EI) *m/z*: 253 (M⁺). Anal. Calcd for C₁₆H₁₅NO₂: C, 75.87; H, 5.97; N, 5.53. Found: C, 75.83; H, 6.12; N, 5.62.

1-[(E)-2-(3,5-Dimethyl-4-nitrophenyl)ethenyl]naphthalene (5): mp 164–165 °C; ¹H NMR (CDCl₃, 400 MHz, δ; ppm) 8.20 (1H, d, *J* = 8 Hz), 7.94–7.83 (3H, m), 7.74 (1H, d, *J* = 7.3 Hz), 7.57–7.49 (3H, m), 7.34 (2H, s), 7.07 (1H, d, *J* = 16.1 Hz), 2.39 (6H, s). MS (EI) *m/z*: 303 (M⁺). Anal. Calcd for C₂₀H₁₇NO₂: C, 79.19; H, 5.65; N, 4.62. Found: C, 79.30; H, 5.67; N, 4.66.

2-[(E)-2-(3,5-Dimethyl-4-nitrophenyl)ethenyl]naphthalene (6): mp 147–149 °C; ¹H NMR (CDCl₃, 400 MHz, δ; ppm) 7.88–7.82 (4H, m), 7.72 (1H, dd, *J* = 1.7, 8.8 Hz), 7.52–7.46 (2H, m), 7.31 (1H, d, *J* = 16.3 Hz), 7.30 (2H, s), 7.14 (1H, d, *J* = 16.34), 2.37 (6H, s). MS (EI) *m/z*: 303 (M⁺). Anal. Calcd for C₂₀H₁₇NO₂: C, 79.19; H, 5.65; N, 4.62. Found: C, 78.96; H, 5.70; N, 4.62.

9-[(E)-2-(3,5-Dimethyl-4-nitrophenyl)ethenyl]anthracene (7): mp 219–221 °C; ¹H NMR (CDCl₃, 400 MHz, δ; ppm) 8.44 (1H, s), 8.31–8.28 (2H, m), 8.05–8.02 (2H, m), 7.98 (1H, d, *J* = 16.6 Hz), 7.52–7.46 (4H, m), 7.42 (2H, s), 6.90 (1H, d, *J* = 16.6 Hz), 2.41 (6H, s). MS (EI) *m/z*: 353 (M⁺). Anal. Calcd for C₂₄H₁₉NO₂: C, 81.56; H, 5.42; N, 3.96. Found: C, 81.70; H, 5.75; N, 3.86.

1,3-Dimethyl-2-nitro-5-(1,3-petadienyl)benzene (8): yellow oil; ¹H NMR (CDCl₃, 400 MHz, δ; ppm) 7.09 (2H, s), 6.80–6.73 (1H, m), 6.33 (1H, d, *J* = 15.8 Hz), 6.25–6.18 (1H, m), 5.94–5.88 (1H, m), 2.31 (6H, s), 1.83 (3H, d, *J* = 6.8 Hz). MS (EI) *m/z*: 217 (M⁺). HRMS: Calcd for C₁₃H₁₅NO₂, 217.110; found, 217.110.

1,3-Dimethyl-2-nitro-5-[(1E,3E)-4-phenyl-1,3-butadienyl]benzene (9): mp 126–130 °C; ¹H NMR (CDCl₃, 400 MHz, δ; ppm) 7.45 (2H, d, *J* = 7.1 Hz), 7.35 (2H, dd, *J* = 7.3, 7.8 Hz), 7.26 (1H, m), 7.17 (2H, s), 7.104–6.903 (2H, m), 6.73 (1H, d, *J* = 14.6 Hz), 6.57 (1H, d, *J* = 14.9 Hz), 2.33 (6H, s). MS (EI) *m/z*: 279 (M⁺). Anal. Calcd for C₁₈H₁₇NO₂: C, 77.40; H, 6.13; N, 5.01. Found: C, 77.17; H, 6.29; N, 5.03.

5-[(E)-2-(2,6-Dimethylphenyl)ethenyl]-1,3-dimethyl-2-nitrobenzene (10): mp 147–149 °C; ¹H NMR (CDCl₃, 400 MHz, δ; ppm) 7.88–7.82 (4H, m), 7.72 (1H, dd, *J* = 1.7, 8.8 Hz), 7.52–7.46 (2H, m), 7.31 (1H, d, *J* = 16.3 Hz), 7.30 (2H, s), 7.14 (1H, d, *J* = 16.3 Hz), 2.37 (6H, s). MS (EI) *m/z*: 303 (M⁺). Anal. Calcd for C₂₀H₁₇NO₂: C, 79.19; H, 5.65; N, 4.62. Found: C, 78.96; H, 5.70; N, 4.62.

5-[(E)-2-(3,5-Dimethoxyphenyl)ethenyl]-1,3-dimethyl-2-nitrobenzene (11): mp 104–105 °C; ¹H NMR (CDCl₃, 400 MHz, δ; ppm) 7.24 (2H, s), 7.07 (1H, d, *J* = 16.4 Hz), 6.99 (1H, d, *J* = 16.4 Hz), 6.67 (2H, d, *J* = 2.2 Hz), 6.43 (1H, t, *J* = 2.2 Hz), 3.85 (6H, s), 2.35 (6H, s); MS (EI) *m/z*: 313 (M⁺). Anal. Calcd for C₁₈H₁₉NO₄: C, 68.99; H, 6.11; N, 4.47. Found: C, 68.79; H, 6.11; N, 4.57.

(E)-3-Methyl-4-nitrostilbene (12): mp 85–87 °C; ¹H NMR (CDCl₃, 400 MHz, δ; ppm) 8.03 (1H, d, *J* = 8.5 Hz), 7.54 (2H, dd, *J* = 1.5, 7.3 Hz), 7.48–7.38 (4H, m), 7.35–7.31 (1H, m), 7.23 (1H, d, *J* = 16.6 Hz), 7.09 (1H, d, *J* = 16.6 Hz), 2.66 (3H, s). MS (EI) *m/z*: 239 (M⁺). Anal. Calcd for C₁₅H₁₃NO₂: C, 75.30; H, 5.48; N, 5.85. Found: C, 75.14; H, 5.44; N, 6.03.

(E)-4-Nitrostilbene (13): mp 161–162 °C; ¹H NMR (CDCl₃, 400 MHz, δ; ppm) 8.22 (2H, ddd, *J* = 2, 2.4, 6.8 Hz), 7.64 (2H, ddd, *J* = 2, 2.4, 8.8 Hz), 7.56 (2H, d, *J* = 7.1 Hz), 7.42–7.39 (2H, m), 7.36–7.31 (1H, m), 7.28 (1H, d, *J* = 16.3 Hz), 7.15 (1H, d, *J* = 16.3 Hz). MS (EI) *m/z*: 225 (M⁺). Anal. Calcd for C₁₄H₁₁NO₂: C, 74.65; H, 4.92; N, 6.22. Found: C, 74.65; H, 4.92; N, 6.22.

(19) Jones, M. E.; Taft, R. W.; Kamlet, M. J. *J. Am. Chem. Soc.* **1977**, *99*, 8452–8453.

ESR Analysis. The Fe^{2+} complex of MGD [Fe^{2+} -MGD₂, (Fe-MGD)] was used to trap NO. Fresh stock solutions of Fe-MGD (1:4) were prepared by adding ferrous ammonium sulfate to an aqueous solution of MGD. A sample containing 1 mM of compounds 1–14 and 20 mM Fe-MGD in distilled water (25% DMSO) was introduced to a quartz flat cuvette cell. ESR spectra were recorded after light irradiation at a distance of 10 cm with a JES-RE 2X spectrometer (JEOL Co. Ltd., Tokyo, Japan). The spectrometer settings were modulation frequency, 100 kHz; modulation amplitude, 2.0 G; scan time, 4 min; microwave power, 16 mW; and microwave frequency, 9.42 GHz.

Griess Assay. The Griess reagents were prepared by mixing acetic acid (5 mL), sulfanilic acid (500 mg), *N*-1-naphthylethylenediamine dihydrochloride (50 mg), and distilled water (95 mL). A sample containing 200 μM compounds 4, 5, 8, 9, 12, or 13 in *o*-dichlorobenzene (2 mL) and distilled water (1 mL) was photoirradiated for the specified times at a distance of 12 cm. The mixture was treated with the Griess reagents (1 mL), and it was agitated for 15 min. The aqueous layer was separated with a fixed angle centrifuge for 5 min at 5000 rpm, and the absorbance at 546 nm was measured. The results were expressed as the percentile of the conversion ratio of the testing compounds.

Cytotoxicity Against HCT116 Cells. HCT116 human colon cancer cells were purchased from American Type Culture Collection (ATCC, Manassas, VA) and cultured in McCoy5A culture medium containing penicillin and streptomycin, supplemented with fetal bovine serum as described in the ATCC instructions. The cells were maintained at 37 °C in a humidified 5% (v/v) CO_2 incubator under sub-confluent conditions. For the experiments, the cells were plated at 2.5×10^5 cells per 6-cm culture dish 2 days before treatments and incubated at 37 °C. On the day of the experiment, the culture medium was refreshed and reduced to 2 mL per 6-cm culture dish. The cells were then treated

with compound 14 in 2 μL of DMSO giving a final concentration of 2.5 μM , which resulted in a final DMSO concentration in the culture media of less than 0.1% (v/v), at which concentration there were no apparent effects from the DMSO. The cells were also treated with compound 14 in the same manner described above in the presence of 40 nM P450 nor (Wako Pure Chemical Industry Co. Ltd, Osaka, Japan) and 2 μM NADH. The treated cells were subsequently subjected to the photoirradiation for 1 min by using the light-source (100 W mercury lamp) of a fluorescence microscope (Olympus BX60/BX-FLA with UplanFl 10X objective lens) with a WU filter (330–380 nm band-pass filter). The irradiation area was set as a circular area with a diameter of around 3 mm. After 24 h incubation, the cells were observed under an inverted phase-contrast microscope, and the images were taken with a digital camera and processed with a personal computer.

MM2 Calculations. Force-field (MM2) minimizations of compounds 1–13 were performed using MacroModel 8.0 software.²¹ All structures were fully optimized with each parameter set as follows: force field, MM2*; method, LBFGS; max no. iterations, 10 000; converge on, gradient; convergence threshold, 0.05.

Supporting Information Available: Ultraviolet–visible-light spectra of compounds 1–14 and the ESR and Griess assay results of compound 14 are reported. This material is available free of charge via the Internet at <http://pubs.acs.org>.

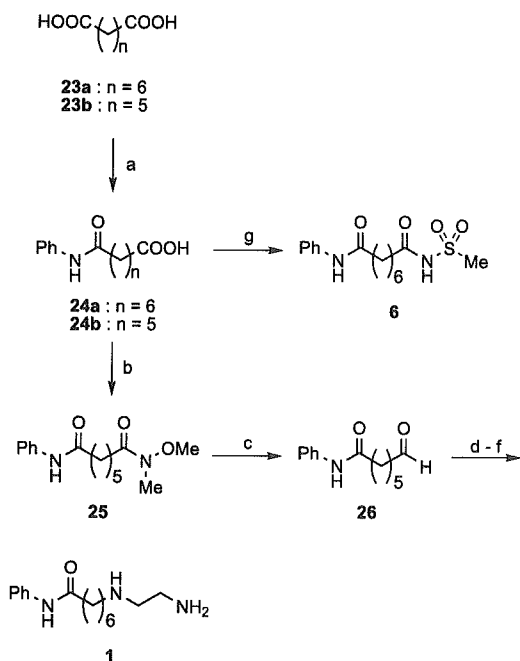
JA0512024

- (20) Goldstein, S. L.; McNelis E. *J. Org. Chem.* **1984**, *49*, 1613–1620.
(21) Mohamadi, F.; Richards, N. G. J.; Guida, W. C.; Liskamp, R.; Lipton, M.; Caufield, C.; Chang, G.; Hendrickson, T.; Still, W. C. *J. Comput. Chem.* **1990**, *11*, 440–467.

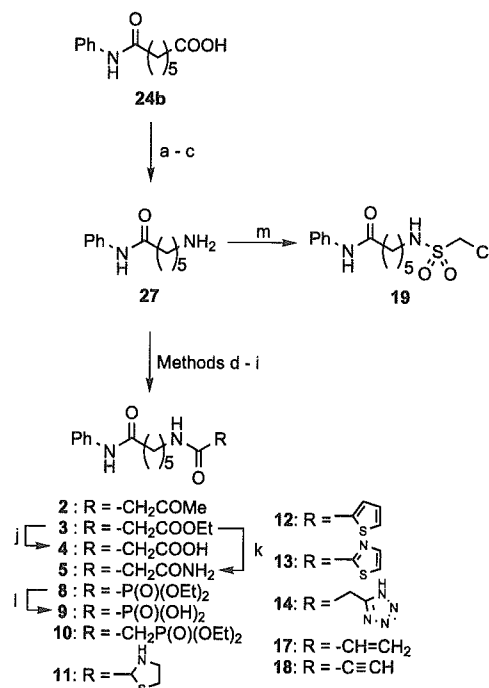
2. Chemistry

The compounds prepared for this study are shown in Table 1. The routes used for synthesis of the compounds are shown in Schemes 1–4. Compounds **1** and **6** were prepared as shown in Scheme 1, starting with dicarboxylic acids **23**. The condensation of aniline gave mono-anilides **24**. The carboxylic acid **24b** was converted to Weinreb amide **25** in the presence of EDCI and HOBT. Compound **25** was allowed to react with lithium aluminum hydride at 0 °C to give aldehyde **26**. Reductive amination of **26** with mono-Boc ethylenediamine, Boc protection of the resultant amine, chromatographic purification, and removal of the two Boc groups afforded ethylenediamine **1**. Carboxylic acid **24a** was converted to acylsulfonamide **6** by acylation of methanesulfonamide in the presence of CDI and DBU in DMF.

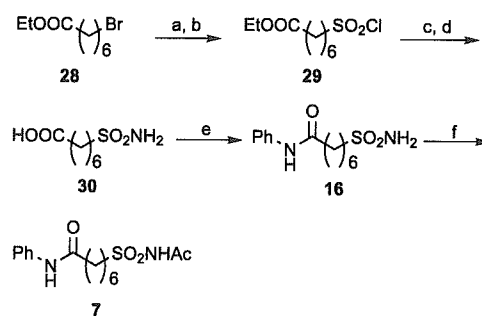
Amine **27** served as a key common intermediate for the synthesis of compounds **2–5**, **8–14**, **17**, and **18** (Scheme 2). Carboxylic acid **24b** was converted to amine **27** by Curtius rearrangement of carboxylic acid **24b**, treatment of the resulting isocyanates with benzyl alcohol, and removal of the Z group by hydrogenolysis. Amine **27** underwent a reaction with diketene to give acetoacetamide **2**. An appropriate carboxylic acid was reacted with amine **27** in the presence of EDCI and HOBT in DMF to give compounds **3**, **12**, **13**, **17**, and **18**. Hydrolysis of ester **3** provided carboxylic acid **4**, and treatment of **3** with 25% aqueous ammonia produced amide **5**. Amine **27** was converted to diethyl carbamoylphosphonate **8**



Scheme 1. Reagents and conditions: (a) aniline, 180 °C; (b) *N,N*-dimethylhydroxylamine hydrochloride, Et₃N, EDCI, HOBT, DMF, rt; (c) LiAlH₄, THF, 0 °C; (d) *N*-(2-aminoethyl)carbamoyl acid *tert*-butyl ester, NaBH(OAc)₃, CH₂Cl₂, AcOH, rt; (e) (Boc)₂O, Et₃N, rt; (f) TFA, CH₂Cl₂, rt; (g) MsNH₂, 1,1'-carbonyldiimidazole, DBU, DMF, rt.

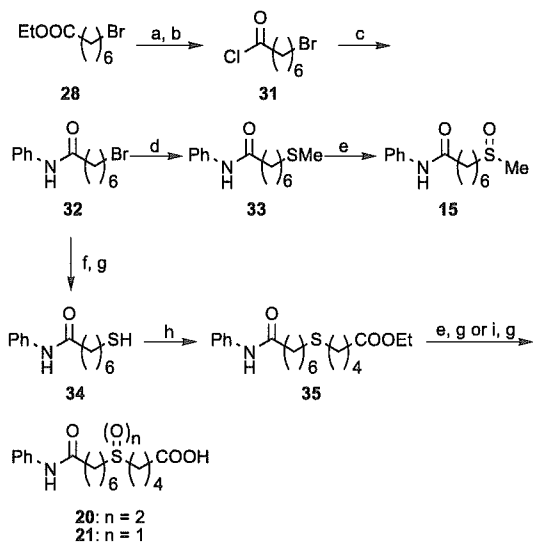


Scheme 2. Reagents and conditions: (a) diphenylphosphoryl azide, Et₃N, benzene, reflux; (b) BnOH, reflux; (c) H₂, 5% Pd-C, MeOH; (d) diketene, rt (for **2**); (e) RCOOH, EDCI, HOBT, DMF, rt (for **3**, **12**, **13**, **17**, and **18**); (f) EtSCOP(O)(OEt)₂, rt (for **8**); (g) (i) bromoacetyl chloride, Et₃N, THF, rt; (ii) P(OEt)₃, tetrabutyl ammonium iodide, reflux (for **10**); (h) (i) thiazolidine-2,3-dicarboxylic acid 3-*tert*-butyl ester, EDCI, HOBT, DMF, rt; (ii) 4NHCl·AcOEt, AcOEt, rt (for **11**); (i) tetrazole-5-acetic acid, bis(2-oxo-3-oxazolidinyl)phosphinic chloride, 0 °C to rt (for **14**); (j) 2 N aq NaOH, MeOH, rt; (k) 25% aq NH₃, MeOH, rt; (l) TMSBr, MeCN, rt; (m) chloromethylsulfonyl chloride, Et₃N, 0 °C to rt.



Scheme 3. Reagents and conditions: (a) Na₂SO₃, EtOH, H₂O, reflux; (b) SOCl₂, DMF, toluene, reflux; (c) 25% aq NH₃, 4-(dimethylamino)pyridine, pyridine, CH₂Cl₂, rt; (d) 2 N aq NaOH, EtOH, rt; (e) aniline, EDCI, HOBT, DMF, rt; (f) AcOH, 1,1'-carbonyldiimidazole, DBU, DMF, rt.

upon reacting with *S*-ethyl diethylphosphonothioformate, and following ester group removal with trimethylsilyl bromide gave carbamoylphosphonic acid **9**. Acylation of amine **27** with bromoacetyl chloride followed by an Arbuzov reaction provided diethyl phosphonate **10**. Coupling between amine **27** and *N*-Boc



Scheme 4. Reagents and conditions: (a) LiOH-H₂O, EtOH, THF, H₂O, rt; (b) (COCl)₂, DMF, CH₂Cl₂, rt; (c) aniline, Et₃N, CH₂Cl₂, rt; (d) 15% aq NaSMe, EtOH, rt; (e) 1 equiv of *m*-chloroperoxybenzoic acid, CH₂Cl₂, rt; (f) AcSK, EtOH, rt; (g) 2 N aq NaOH, EtOH, rt; (h) 5-bromovaleric acid ethyl ester, NaOEt, EtOH, rt; (i) 2 equiv of *m*-chloroperoxybenzoic acid, CH₂Cl₂, rt.

thiazolidine-2-carboxylic acid in the presence of EDCI and HOBT and subsequent deprotection of the Boc group under acidic conditions produced 2-thiazolidinecarboxamide **11**. Amine **27** was reacted with tetrazole-5-acetic acid in the presence of bis(2-oxo-3-oxazolidinyl)phosphinic chloride to give tetrazole-5-acetamide **14**. Chloromethyl sulfonamide **19** was obtained by the coupling of amine **27** with chlorosulfonyl chloride.

The preparation of sulfonamide derivatives **7** and **16** was achieved via sulfonyl chloride **29** (Scheme 3). Bromide **28** was converted to sulfonyl chloride **29** by a reaction with Na₂SO₃ and by subsequent treatment with thionyl chloride. Treatment of the sulfonyl chloride **29** with 25% aqueous ammonia and subsequent hydrolysis yielded sulfonamide **30**. The condensation of carboxylic acid **30** with aniline gave compound **16**. Sulfonamide **16** was acetylated to the reversed acylsulfonamide **7** in the same procedure employed in the preparation of **6**.

Compounds **15**, **20**, and **21** were synthesized from ester **28** as depicted in Scheme 4. Acid chloride **31** was prepared from ester **28** by hydrolysis of the ethyl ester and subsequent reaction with oxalyl chloride. The amino group of aniline was acylated with the acid chloride **31** to give the amide **32**. Sulfide **33** was obtained by the alkylation of methylmercaptan with bromide **32**. Oxidation of **33** with 1 equiv of MCPBA gave the sulfoxide **15**. Bromide **32** was also treated with potassium thioacetate to give the thioester, after which deacetylation of the thioacetate under alkaline conditions gave thiol **34**. Thiol **34** was alkylated with ethyl 5-bromovalerate, oxidized with 1 or 2 equivalent amounts of MCPBA, and then hydrolyzed to give compounds **20** and **21**.

3. Results and discussion

3.1. Enzyme assays

The compounds synthesized in this study were tested with an in vitro assay using a HeLa nuclear extract rich in HDAC activity. The results are summarized in Table 1. SAHA was used as a positive control, which inhibited 100% of the HDAC activity at 100 μM and had an IC₅₀ of 0.28 μM.

Table 1. HDAC enzyme inhibition data for compounds **1–22**^a

Compound	R	n	% inhibition at 100 μM
1	-NHCH ₂ CH ₂ NH ₂	6	12 ± 1.2
2	-NHCOCH ₂ COMe	5	12 ± 1.3
3	-NHCOCH ₂ COOEt	5	0 ± 1.4
4	-NHCOCH ₂ COOH	5	4.1 ± 0.56
5	-NHCOCH ₂ CONH ₂	5	1.1 ± 2.2
6	-CONHSO ₂ Me	6	21 ± 3.0
7	-SO ₂ NHAc	6	2.5 ± 1.9
8	-NHCOP(O)(OEt) ₂	5	0 ± 1.6
9	-NHCOP(O)(OH) ₂	5	5.8 ± 1.3
10	-NHCOCH ₂ P(O)(OEt) ₂	5	0 ± 0.36
11		5	0 ± 1.5
12		5	21 ± 4.6
13		5	2.0 ± 1.1
14		5	17 ± 1.7
15	-S(O)Me	6	62 ± 2.0
16	-SO ₂ NH ₂	6	10 ± 0.97
17	-NHCOCH=CH ₂	5	11 ± 3.5
18	-NHCOC≡CH	5	3.7 ± 2.3
19	-NHSO ₂ CH ₂ Cl	5	0 ± 0.56
20	-SO ₂ (CH ₂) ₄ COOH	6	6.1 ± 3.3
21	-S(O)(CH ₂) ₄ COOH	6	12 ± 6.1
22	-SO ₂ Me	6	33 ± 2.0 ^b

^a Values are means ± standard deviations of a minimum of three separate experiments.

^b Data taken from the literature (Ref. 10i).

The co-crystal structure of an archaeobacterial HDAC homologue (HDAC-like protein, HDLP) with SAHA revealed that the hydroxamic acid group coordinates the zinc ion in the active site through its CO and OH groups.⁷ Based on these data, we designed compounds **1–14** which were expected to chelate zinc ion in the active site in a bidentate fashion. Notably, an acylsulfonamide moiety has been reported to bind zinc ion in a bidentate fashion through its CO and SO₂ groups,¹¹ and carbamoylphosphonic acids are known to be matrix

metalloproteinase inhibitors,¹² another form of zinc-dependent metalloenzyme. However, these compounds were found to be totally inactive, although acylsulfonamide **6** and 2-thiophenecarboxamide **12** showed a certain level of inhibitory activity against HDACs. The reason that compounds **1–14** were weakly active is unclear, but it is probably because they lack interaction with amino acid residues such as Tyr and His in the active site of HDACs.⁷

Next, compounds with methyl sulfoxide (**15**) and sulfonamide¹³ (**16**), which are expected to coordinate zinc ions monodentately, were evaluated for anti-HDAC activity. Interestingly, although the inhibitory ability of monodentate zinc-binding groups (ZBGs) was thought to be less than that of bidentate ZBGs, methyl sulfoxide **15** was more potent than compounds **1–14** in inhibiting 68% of the HDAC activity at 100 μM , with an IC_{50} of 48 μM .

The crystal structure of the HDLP/SAHA complex made it clear that there are nucleophilic amino acids such as His in the active center of HDACs.⁷ Compounds bearing acrylamide (**17**), 2-propynamide (**18**), and chloromethanesulfonamide (**19**) could form covalent bonds with nucleophilic amino acids of the enzyme and function as irreversible HDAC inhibitors. However, these compounds were only weakly active against HDACs.

According to the data of the crystal structure of the HDLP/SAHA complex, there is a 14 Å long internal cavity adjacent to the active site.^{7,14} We designed the cavity-targeting compounds **20** and **21** modeled after sulfoxide **15** and sulfone **22**. Sulfone **22** has been reported to inhibit HDACs.¹⁰ⁱ The carboxylic acid of **20** and **21** could interact with Arg 27 (HDLP numbering), a component in the construction of the 14 Å internal cavity, and these compounds were anticipated to be more potent HDAC inhibitors. However, contrary to expectation, compounds **20** and **21** did not show strong activity as compared with their parent compounds **15** and **22**. At the time this work was ongoing, the X-ray structure of human HDAC8 was published.¹⁵ According to this work, there is no such large internal cavity adjacent to the active site. This may be the reason why compounds **20** and **21** did not exhibit inhibitory activity against human HDACs.

3.2. Cellular assays

HDACs are also responsible for the deacetylation of non-histone proteins.^{1c} Notably, HDAC6, one of the isozymes of HDAC, has recently been reported to be a α -tubulin deacetylase (TDAC).¹⁶ The reversible acetylation of α -tubulin is involved in microtubule stability. Therefore, compounds with high HDAC/TDAC selectivity are desirable for biological studies and medicinal use. However, many hydroxamate HDAC inhibitors such as TSA and SAHA cause the accumulation of acetylated histones as well as acetylated α -tubulin, which indicates that they do not discriminate between HDAC6 and the other isozymes.⁹ To investigate the selectivity of sulfoxide **15**, the most active compound in this study,



Figure 2. Western blot analysis of histone hyperacetylation and α -tubulin acetylation induction in HCT 116 cells produced by compound **15** and by reference compound SAHA.

Western blot analysis was performed (Fig. 2). HCT116 human colon cancer cells were treated with SAHA or compound **15** for 8 h at 37 °C. As reported previously, 5 μM of SAHA caused the accumulation of both acetylated histone H4 and acetylated α -tubulin. On the other hand, 100 μM of compound **15** did not induce α -tubulin acetylation although the same level of histone H4 hyperacetylation as with SAHA was observed. These results suggested that compound **15** is inactive toward TDAC (HDAC6) in cells.

4. Conclusion

We have designed and prepared a series of SAHA-based non-hydroxamate compounds as (i) analogues bearing functional groups expected to chelate zinc ion bidentately (compounds **1–14**) or monodentately (compounds **15** and **16**), (ii) irreversible inhibition-oriented compounds (compounds **17–19**), and (iii) 14 Å internal cavity-targeting compounds (compounds **20** and **21**), and evaluated their inhibitory effect on HDACs. Among them, methyl sulfoxide **15** inhibited HDACs in enzyme assays and showed great HDAC/TDAC selectivity in cellular assays.

In conclusion, we have identified a novel lead compound, from which potent HDAC isozyme-selective inhibitors can be developed. The findings of this study should also pave the way for the development of new medicines without side effects caused by interference with microtubule dynamics associated with HDAC6. Currently, detailed structure–activity relationship studies of methyl sulfoxide-based HDAC inhibitors are under way.

5. Experimental section

5.1. Chemistry

Melting points were determined using a Yanagimoto micro melting point apparatus or a Büchi 545 melting point apparatus and were left uncorrected. Proton nuclear magnetic resonance spectra (¹H NMR) were recorded on a JEOL JNM-LA400 or JEOL JNM-LA500 spectrometer in solvent as indicated. Chemical shifts (δ) were reported in parts per million relative to the internal standard tetramethylsilane. Elemental analysis was performed with a Yanaco CHN CORDER NT-5 analyzer, and all values were within $\pm 0.4\%$ of the

calculated values. High-resolution mass spectra (HRMS) were recorded on a JEOL JMS-SX102A mass spectrometer. GC–MS analyses were performed on a Shimadzu GCMS-QP2010. Reagents and solvents were purchased from Aldrich, Tokyo Kasei Kogyo, Wako Pure Chemical Industries, and Kanto Kagaku and used without purification. Flash column chromatography was performed using Silica Gel 60 (particle size 0.046–0.063 mm) supplied by Merck.

5.1.1. 6-Phenylcarbamoylhexanoic acid (24b). A mixture of aniline (5.80 g, 62.3 mmol) and pimeric acid (**23b**, 10.0 g, 62.4 mmol) was stirred at 180 °C for 1 h. After cooling, the mixture was diluted with AcOEt–THF and the slurry was filtered. The filtrate was washed with saturated aqueous NaHCO₃ and the aqueous layer was acidified with concentrated HCl. The precipitated crystals were collected by filtration to give 7.11 g (49%) of **24b** as a white solid: ¹H NMR (DMSO-*d*₆ 400 MHz, δ; ppm) 11.97 (1H, broad s), 9.83 (1H, s), 7.58 (2H, d, *J* = 7.8 Hz), 7.27 (2H, t, *J* = 7.9 Hz), 7.01 (1H, t, *J* = 7.4 Hz), 2.67 (2H, t, *J* = 7.4 Hz), 2.21 (2H, t, *J* = 7.3 Hz), 1.62–1.49 (4H, m), 1.34–1.27 (2H, m).

5.1.2. Heptanedioic acid methoxymethylamide phenylamide (25). To a solution of **24b** (5.7 g, 24.2 mmol) obtained above and *N,O*-dimethylhydroxylamine hydrochloride (4.80 g, 49.2 mmol) in DMF (70 mL) were added 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (6.00 g, 31.3 mmol), 1-hydroxy-1*H*-benzotriazole monohydrate (4.80 g, 31.3 mmol), and triethylamine (7.50 mL, 54.0 mmol) and the mixture was stirred overnight at room temperature. The reaction mixture was poured into water and extracted with AcOEt. The AcOEt layer was separated, washed with water, 2 N aqueous HCl, saturated aqueous NaHCO₃ and brine, and was dried over Na₂SO₄. Filtration and concentration in vacuo and separation by silica gel flash chromatography (*n*-hexane/AcOEt = 8:1) gave 6.34 g (94%) of **25** as a colorless crystal: ¹H NMR (CDCl₃, 400 MHz, δ; ppm) 7.54 (2H, d, *J* = 7.8 Hz), 7.41 (1H, broad s), 7.31 (2H, t, *J* = 7.6 Hz), 7.09 (1H, t, *J* = 7.6 Hz), 3.67 (3H, s), 3.18 (3H, s), 2.45 (2H, t, *J* = 7.3 Hz), 2.39 (2H, t, *J* = 7.3 Hz), 1.77 (2H, quintet, *J* = 7.3 Hz), 1.70 (2H, quintet, *J* = 7.6 Hz), 1.44 (2H, quintet, *J* = 7.8 Hz).

5.1.3. 7-Oxoheptanoic acid phenylamide (26). To a suspension of lithium aluminum hydride (300 mg, 7.91 mmol) in THF (30 mL) was added a solution of **25** (2.50 g, 8.98 mmol) obtained above in THF (20 mL) dropwise with cooling by an ice-water bath. The reaction mixture was stirred at 0 °C for 30 min. To the mixture were added water (0.3 mL), 15% aqueous NaOH (0.3 mL), and water (0.9 mL) in a sequential order and the slurry was filtered. After the solid was washed with THF (10 mL), the combined filtrates were concentrated in vacuo. The residue was purified by silica gel flash chromatography (*n*-hexane/AcOEt = 1:1) to give 1.42 g (72%) of **26** as a white solid: ¹H NMR (CDCl₃, 400 MHz, δ; ppm) 9.77 (1H, s), 7.52 (2H, d, *J* = 8.1 Hz), 7.32 (2H, t, *J* = 7.6 Hz), 7.25 (1H, broad s), 7.10 (1H, t, *J* = 7.3 Hz), 2.47 (2H, t, *J* = 7.1 Hz),

2.37 (2H, t, *J* = 7.3 Hz), 1.76 (2H, quintet, *J* = 7.3 Hz), 1.68 (2H, quintet, *J* = 7.4 Hz), 1.42 (2H, quintet, *J* = 7.3 Hz).

5.1.4. 7-(2-Aminoethylamino)heptanoic acid phenylamide ditrifluoroacetic acid salt (1-2TFA). To a solution of **26** (240 mg, 1.09 mmol) obtained above in CH₂Cl₂ (3 mL) and AcOH (1 mL) was added *N*-(2-aminoethyl)carbamamic acid *tert*-butyl ester (190 mg, 1.19 mmol) at room temperature. The solution was stirred for 15 min, followed by the addition of sodium triacetoxy borohydride (475 mg, 2.24 mmol). The solution was stirred overnight at room temperature. To the solution were added triethylamine (12 mL) and di-*tert*-butyl dicarbonate (300 mg, 1.37 mmol), and the mixture was stirred at room temperature for 5 h. The reaction mixture was poured into water and extracted with AcOEt. The AcOEt layer was separated, washed with water, saturated aqueous NaHCO₃ and brine, and was dried over Na₂SO₄. Filtration and concentration in vacuo and purification by silica gel flash chromatography (*n*-hexane/AcOEt = 5:2) gave 120 mg of colorless oil. To a solution of the oil in CH₂Cl₂ (2 mL) was added trifluoroacetic acid (3 mL), and the mixture was stirred at room temperature for 1 h. The solution was concentrated in vacuo to give a solid. The residue was triturated in ether to give 112 mg (21%) of **1-2TFA** as a crude solid. The solid was recrystallized from AcOEt and collected by filtration to give 103 mg of **1-2TFA** as colorless crystals: mp 113–114 °C; ¹H NMR (DMSO-*d*₆ 400 MHz, δ; ppm) 9.87 (1H, s), 7.58 (2H, d, *J* = 7.8 Hz), 7.28 (2H, t, *J* = 7.6 Hz), 7.02 (1H, t, *J* = 7.1 Hz), 3.20–3.05 (4H, m), 2.95 (2H, t, *J* = 8.1 Hz), 2.31 (2H, t, *J* = 7.1 Hz), 1.66–1.50 (4H, m), 1.42–1.26 (4H, m); Anal. Calcd for C₁₅H₂₅N₃O₂TFA: C, 46.44; H, 5.54; N, 8.55. Found: C, 46.25; H, 5.44; N, 8.27.

5.1.5. Phenylcarbamoylheptanoic acid (24a). Compound **24a** was prepared from aniline and suberic acid in 51% yield by using the same procedure described for **24b**: ¹H NMR (DMSO-*d*₆ 400 MHz, δ; ppm) 11.98 (1H, broad s), 9.84 (1H, s), 7.58 (2H, d, *J* = 7.8 Hz), 7.28 (2H, t, *J* = 7.8 Hz), 7.01 (1H, t, *J* = 7.3 Hz), 2.29 (2H, t, *J* = 7.4 Hz), 2.20 (2H, t, *J* = 7.3 Hz), 1.58 (2H, t, *J* = 7.2 Hz), 1.50 (2H, t, *J* = 7.1 Hz), 1.29 (4H, m).

5.1.6. 8-Methanesulfonylamino-8-oxooctanoic acid phenylamide (6). To a solution of **24a** (500 mg, 2.01 mmol) in DMF (5 mL) was added 1,1-carbonyldiimidazole (360 mg, 2.22 mmol). After 1 h, methanesulfonamide (210 mg, 2.21 mmol) and 1,8-diazabicyclo[5.4.0]-7-undecene (330 μL, 2.21 mmol) were added to the reaction mixture. After overnight stirring, the mixture was poured into 2 N aqueous HCl and the precipitated crystals were collected by filtration to give 595 mg (91%) of **6** as a crude solid. The solid was recrystallized from AcOEt and collected by filtration to give 511 mg of **6** as colorless crystals: mp 145–146 °C; ¹H NMR (DMSO-*d*₆ 400 MHz, δ; ppm) 11.64 (1H, broad s), 9.84 (1H, broad s), 7.58 (2H, d, *J* = 8.0 Hz), 7.28 (2H, t, *J* = 7.8 Hz), 7.01 (1H, t, *J* = 7.1 Hz), 3.22 (3H, s), 2.29 (2H, t, *J* = 7.3 Hz), 2.26 (2H, t, *J* = 7.3 Hz), 1.58 (2H, quintet, *J* = 7.4 Hz), 1.52 (2H, quintet,

$J = 7.3$ Hz), 1.36–1.22 (4H, m); MS (EI) m/z : 326 (M^+); Anal. Calcd for $C_{15}H_{22}N_2O_4S$: C, 55.19; H, 6.79; N, 8.58. Found: C, 55.00; H, 6.72; N, 8.73.

5.1.7. 6-Aminohexanoic acid phenylamide (27). To a suspension of **24b** (1.11 g, 4.73 mmol) and triethylamine (699 mg, 6.90 mmol) in benzene (3 mL) was added diphenylphosphoryl azide (1.83 g, 6.64 mmol) and the mixture was heated at reflux temperature for 1 h. Next, benzyl alcohol (1.20 mL, 11.6 mmol) was added and the reaction mixture was stirred at reflux temperature for 24 h. It was then concentrated in vacuo and the residue was dissolved in AcOEt. The AcOEt solution was washed with 0.4 N aqueous HCl, water, saturated aqueous $NaHCO_3$, and brine, and was dried over Na_2SO_4 . Filtration and concentration in vacuo, and purification by recrystallization from $CHCl_3$ -*n*-hexane gave 1.01 g (63%) of (6-phenylcarbamoylpentyl)carbamate acid benzyl ester as a colorless needle: 1H NMR (DMSO- d_6 , 400 MHz, δ ; ppm) 9.81 (1H, s), 7.57 (2H, d, $J = 7.8$ Hz), 7.37–7.22 (8H, m), 7.00 (1H, t, $J = 7.4$ Hz), 4.99 (2H, s), 2.99 (2H, q, $J = 6.5$ Hz), 2.28 (2H, t, $J = 7.4$ Hz), 1.58 (2H, quintet, $J = 7.6$ Hz), 1.43 (2H, quintet, $J = 7.1$ Hz), 1.32 (2H, quintet, $J = 7.8$ Hz); MS (EI) m/z : 340 (M^+).

A solution of (6-phenylcarbamoylpentyl)carbamate acid benzyl ester (1.00 g, 2.95 mmol) obtained above in MeOH (50 mL) was stirred under H_2 (atmospheric pressure) in the presence of 5% Pd/C (106 mg) at room temperature for 7 h. The catalyst was removed by filtration through Celite, and the filtrate was concentrated in vacuo. The residue was purified by silica gel flash chromatography ($CHCl_3$ /MeOH/*i*-PrNH $_2$ = 19:1:1) to give 584 mg (96%) of **27** as a white solid: 1H NMR (DMSO- d_6 , 400 MHz, δ ; ppm) 9.83 (1H, s), 7.58 (2H, d, $J = 7.6$ Hz), 7.27 (2H, t, $J = 7.9$ Hz), 7.01 (1H, t, $J = 7.3$ Hz), 2.55 (2H, m), 2.29 (2H, t, $J = 7.4$ Hz), 1.59 (2H, quintet, $J = 7.4$ Hz), 1.37–1.30 (4H, m).

5.1.8. 6-(3-Oxobutylamino)hexanoic acid phenylamide (2). To a solution of **27** (107 mg, 0.52 mmol) obtained above in MeOH (3 mL) was added diketene (0.3 mL, 3.89 mmol) at 0 °C and the resulting mixture was stirred at room temperature for 9 h. The solution was concentrated in vacuo to give a crude solid. The solid was purified by silica gel flash column chromatography (AcOEt only to AcOEt/MeOH = 9:1) to give 108 mg (72%) of **2** as a yellow solid. The solid (108 mg) was recrystallized from *n*-hexane–AcOEt–MeOH and collected by filtration to give 69 mg of **2** as brown crystals: mp 126–128 °C; 1H NMR (DMSO- d_6 , 500 MHz, δ ; ppm) 9.85 (1H, s), 8.02 (1H, s), 7.56 (2H, d, $J = 8.2$ Hz), 7.28 (2H, t, $J = 7.9$ Hz), 7.01 (1H, t, $J = 7.3$ Hz), 3.27 (2H, s), 3.05 (2H, q, $J = 6.5$ Hz), 2.29 (2H, t, $J = 7.5$ Hz), 2.12 (3H, s), 1.58 (2H, quintet, $J = 7.3$ Hz), 1.43 (2H, quintet, $J = 7.5$ Hz), 1.30 (2H, quintet, $J = 7.8$ Hz); MS (EI) m/z : 290 (M^+); Anal. Calcd for $C_{16}H_{22}N_2O_3$: C, 66.18; H, 7.64; N, 9.65. Found: C, 66.04; H, 7.40; N, 9.48.

Compounds **3**, **12**, **13**, **17**, and **18** were prepared from amine **27** and an appropriate carboxylic acid using the procedure described for **25**.

5.1.9. *N*-(5-Phenylcarbamoylpentyl)malonamic acid ethyl ester (3). Mp 107–110 °C; 1H NMR ($CDCl_3$, 500 MHz, δ ; ppm) 7.52 (2H, d, $J = 8.2$ Hz), 7.32 (2H, t, $J = 7.9$ Hz), 7.28 (1H, broad s), 7.18 (1H, broad s), 7.10 (1H, t, $J = 7.3$ Hz), 4.19 (2H, q, $J = 7.1$ Hz), 3.31 (2H, q, $J = 6.6$ Hz), 3.29 (2H, s), 2.37 (2H, t, $J = 7.5$ Hz), 1.77 (2H, quintet, $J = 7.5$ Hz), 1.59 (2H, quintet, $J = 7.3$ Hz), 1.43 (2H, quintet, $J = 7.5$ Hz), 1.28 (3H, t, $J = 7.2$ Hz); MS (EI) m/z : 320 (M^+); Anal. Calcd for $C_{17}H_{24}N_2O_4$: C, 63.73; H, 7.55; N, 8.74. Found: C, 63.82; H, 7.65; N, 8.47.

5.1.10. Thiophene-2-carboxylic acid (5-phenylcarbamoylpentyl)amide (12). Mp 139–141 °C; 1H NMR ($CDCl_3$, 500 MHz, δ ; ppm) 7.53 (2H, d, $J = 7.6$ Hz), 7.52 (1H, d, $J = 4.9$ Hz), 7.45 (1H, d, $J = 4.9$ Hz), 7.35 (1H, broad s), 7.31 (2H, t, $J = 7.9$ Hz), 7.10 (1H, t, $J = 7.3$ Hz), 7.04 (1H, t, $J = 4.9$ Hz), 6.27 (1H, broad s), 3.47 (2H, q, $J = 6.6$ Hz), 2.40 (2H, t, $J = 7.2$ Hz), 1.80 (2H, quintet, $J = 7.4$ Hz), 1.67 (2H, quintet, $J = 7.0$ Hz), 1.48 (2H, quintet, $J = 7.7$ Hz); MS (EI) m/z : 316 (M^+); HRMS Calcd for $C_{17}H_{20}N_2O_2S$ 316.125. Found 316.125.

5.1.11. Thiazole-2-carboxylic acid (5-phenylcarbamoylpentyl)amide (13). Mp 130–133 °C; 1H NMR (DMSO- d_6 , 500 MHz, δ ; ppm) 9.84 (1H, broad s), 8.85 (1H, broad s), 8.02 (1H, d, $J = 3.4$ Hz), 7.99 (1H, d, $J = 3.1$ Hz), 7.57 (2H, d, $J = 7.9$ Hz), 7.27 (2H, t, $J = 7.9$ Hz), 7.01 (1H, t, $J = 7.0$ Hz), 3.26 (2H, q, $J = 6.7$ Hz), 2.30 (2H, t, $J = 7.5$ Hz), 1.59 (2H, quintet, $J = 7.6$ Hz), 1.56 (2H, quintet, $J = 7.9$ Hz), 1.32 (2H, quintet, $J = 7.5$ Hz); MS (EI) m/z : 317 (M^+); HRMS Calcd for $C_{16}H_{19}N_3O_2S$ 317.120. Found 317.119.

5.1.12. *N*-(5-Phenylcarbamoylpentyl)acrylamide (17). Mp 166–168 °C; 1H NMR (DMSO- d_6 , 500 MHz, δ ; ppm) 9.84 (1H, s), 8.07 (1H, s), 7.58 (2H, d, $J = 7.9$ Hz), 7.27 (2H, t, $J = 7.9$ Hz), 7.01 (1H, t, $J = 7.5$ Hz), 6.20 (1H, dd, $J = 10.1, 17.1$ Hz), 6.05 (1H, dd, $J = 2.2, 17.1$ Hz), 5.55 (1H, dd, $J = 2.2, 10.1$ Hz), 3.12 (2H, q, $J = 5.8$ Hz), 2.29 (2H, t, $J = 7.5$ Hz), 1.59 (2H, quintet, $J = 7.5$ Hz), 1.45 (2H, quintet, $J = 7.9$ Hz), 1.30 (2H, quintet, $J = 7.3$ Hz); MS (EI) m/z : 260 (M^+); Anal. Calcd for $C_{15}H_{20}N_2O_2$: C, 69.20; H, 7.74; N, 10.76. Found: C, 68.97; H, 7.76; N, 10.64.

5.1.13. Propynoic acid (5-phenylcarbamoylpentyl)amide (18). Mp 143–145 °C; 1H NMR (DMSO- d_6 , 500 MHz, δ ; ppm) 9.84 (1H, s), 8.70 (1H, s), 7.58 (2H, d, $J = 7.9$ Hz), 7.28 (2H, t, $J = 7.9$ Hz), 7.01 (1H, t, $J = 7.3$ Hz), 4.09 (1H, s), 3.07 (2H, q, $J = 6.7$ Hz), 2.29 (2H, t, $J = 7.3$ Hz), 1.58 (2H, quintet, $J = 7.3$ Hz), 1.43 (2H, quintet, $J = 7.6$ Hz), 1.28 (2H, quintet, $J = 7.3$ Hz); MS (EI) m/z : 258 (M^+); HRMS Calcd for $C_{15}H_{18}N_2O_2$: 258.137. Found: 258.137.

5.1.14. *N*-(5-Phenylcarbamoylpentyl)malonamic acid (4). To a solution of **3** (134 mg, 0.42 mmol) in MeOH (3 mL) was added 2 N aqueous NaOH (0.5 mL, 1.00 mmol), and the mixture was stirred at room temperature for 30 min. The reaction mixture was concentrated and dissolved in AcOEt. The solution was washed with 2 N aqueous HCl and brine, and was dried over Na_2SO_4 . Filtration and

concentration in vacuo gave a crude solid. The solid was recrystallized from *n*-hexane–AcOEt and collected by filtration to give 54 mg (43%) as colorless crystals: mp 128–130 °C; ¹H NMR (DMSO-*d*₆, 500 MHz, δ; ppm) 9.84 (1H, s), 8.32 (1H, s), 8.02 (1H, s), 7.58 (2H, d, *J* = 7.6 Hz), 7.28 (2H, t, *J* = 7.9 Hz), 7.01 (1H, t, *J* = 7.5 Hz), 3.10 (2H, s), 3.05 (2H, q, *J* = 6.4 Hz), 2.29 (2H, t, *J* = 7.5 Hz), 1.59 (2H, quintet, *J* = 7.5 Hz), 1.43 (2H, quintet, *J* = 7.3 Hz), 1.30 (2H, quintet, *J* = 7.6 Hz); Anal. Calcd for C₁₅H₂₀N₂O₄: C, 61.63; H, 6.90; N, 9.58. Found: C, 61.67; H, 6.95; N, 9.47.

5.1.15. *N*-(5-Phenylcarbamoylpentyl)acrylamide (5). To a solution of **3** (61 mg, 0.19 mmol) in MeOH (1.5 mL) was added 25% aqueous NH₃ (1.5 mL), and the mixture was stirred overnight at room temperature. The reaction mixture was concentrated in vacuo and the residue was triturated in AcOEt to give 16 mg (28%) of **5** as a crude solid. The solid was recrystallized from *n*-hexane–AcOEt–MeOH and collected by filtration to give 13 mg of **5** as colorless crystals: mp 166–168 °C; ¹H NMR (DMSO-*d*₆, 500 MHz, δ; ppm) 9.84 (1H, s), 7.97 (1H, t, *J* = 5.3 Hz), 7.58 (2H, d, *J* = 7.9 Hz), 7.41 (1H, broad s), 7.28 (2H, t, *J* = 7.9 Hz), 7.01 (1H, t, *J* = 7.31 Hz), 6.99 (1H, broad s), 3.05 (2H, q, *J* = 6.5 Hz), 2.97 (2H, s), 2.28 (2H, t, *J* = 7.6 Hz), 1.58 (2H, quintet, *J* = 7.6 Hz), 1.43 (2H, quintet, *J* = 7.2 Hz), 1.30 (2H, quintet, *J* = 7.5 Hz); MS (EI) *m/z*: 291 (M⁺); Anal. Calcd for C₁₅H₂₁N₃O₃: C, 61.84; H, 7.27; N, 14.42. Found: C, 61.68; H, 7.25; N, 14.17.

5.1.16. (5-Phenylcarbamoylpentylcarbamoyl)phosphonic acid diethyl ester (8). Under Ar atmosphere, to a solution of ethyl chloroformate (1.14 g, 9.12 mmol) in dry toluene (50 mL) was slowly added triethylphosphite (2.82 g, 17.0 mmol) at 0 °C and the resulting mixture was stirred at room temperature for 4 h. The solution was concentrated and purified by silica gel flash column chromatography (AcOEt/*n*-hexane = 1:2) to give 1.25 g (61%) of *S*-ethyl diethylphosphonothioformate as a crude oil: ¹H NMR (CDCl₃, 500 MHz, δ; ppm) 4.25 (4H, m), 3.01 (2H, q, *J* = 8.5 Hz), 1.37 (6H, t, *J* = 5.5 Hz), 1.28 (3H, t, *J* = 9.3 Hz); MS (EI) *m/z* 226 (M⁺).

To a solution of *S*-ethyl diethylphosphonothioformate (679 mg, 3.00 mmol) obtained above in MeCN (5 mL) was added **27** (215 mg, 1.04 mmol), and the solution was stirred overnight at room temperature. The solution was concentrated and purified by silica gel flash column chromatography (AcOEt/*n*-hexane = 1/2 to AcOEt only) to give 208 mg (54%) of **8** as a colorless oil: ¹H NMR (CDCl₃, 500 MHz, δ; ppm) 7.65 (1H, broad s), 7.55 (2H, d, *J* = 7.9 Hz), 7.30 (2H, t, *J* = 7.9 Hz), 7.22 (1H, broad s), 7.09 (1H, t, *J* = 7.5 Hz), 4.22 (4H, m), 3.34 (2H, q, *J* = 6.6 Hz), 2.36 (2H, t, *J* = 7.3 Hz), 1.75 (2H, quintet, *J* = 7.6 Hz), 1.59 (2H, quintet, *J* = 7.5 Hz), 1.41 (2H, quintet, *J* = 7.6 Hz), 1.36 (6H, t, *J* = 7.2 Hz); MS (EI) *m/z*: 370 (M⁺); HRMS Calcd for C₁₇H₂₇N₂O₅P 370.166. Found 370.165.

5.1.17. (5-Phenylcarbamoylpentylcarbamoyl)phosphonic acid (9). A solution of **8** (156 mg, 0.42 mmol) and bromotrimethylsilane (614 mg, 4.01 mmol) in MeCN

(5 mL) was stirred overnight at room temperature. After the addition of MeOH (1 mL), the solution was concentrated in vacuo and the residue was triturated in AcOEt–MeOH–*n*-hexane to give 57 mg (43%) of **9** as a crude solid. The solid was recrystallized from AcOEt–MeOH and collected by filtration to give 53 mg of **9** as colorless crystals: mp 154–156 °C; ¹H NMR (DMSO-*d*₆, 500 MHz, δ; ppm) 9.85 (1H, s), 8.35 (1H, broad s), 7.58 (2H, d, *J* = 8.2 Hz), 7.28 (2H, t, *J* = 8.1 Hz), 7.01 (1H, t, *J* = 7.3 Hz), 3.10 (2H, q, *J* = 6.7 Hz), 2.29 (2H, t, *J* = 7.6 Hz), 1.59 (2H, quintet, *J* = 7.6 Hz), 1.46 (2H, quintet, *J* = 7.6 Hz), 1.27 (2H, quintet, *J* = 7.6 Hz); Anal. Calcd for C₁₃H₁₉N₂O₅P: C, 49.68; H, 6.09; N, 8.91. Found: C, 49.51; H, 6.06; N, 8.82.

5.1.18. [(5-Phenylcarbamoylpentylcarbamoyl)methyl]-phosphonic acid diethyl ester (10). A mixture of **27** (618 mg, 3.00 mmol), Et₃N (1.0 mL, 7.20 mmol), and bromoacetyl chloride (1.0 mL, 6.5 mmol) in THF (20 mL) was stirred at room temperature for 1 h. The reaction mixture was diluted with CHCl₃. The solution was washed with saturated aqueous NaHCO₃, water, and brine, and was dried over Na₂SO₄. Filtration and concentration in vacuo, and purification by silica gel flash column chromatography gave 693 mg (71%) of a 1:1 mixture of bromoacetyl 6-anilino-6-oxohexanamide and chloroacetyl 6-anilino-6-oxohexanamide as a light yellow solid: ¹H NMR (CDCl₃, 400 MHz, δ; ppm) 7.52 (2H, d, *J* = 8.1 Hz), 7.32 (2H, t, *J* = 7.8 Hz), 7.10 (1H, t, *J* = 7.3 Hz), 6.64, 6.57 (1H, broad s), 4.03, 3.86 (2H, s), 3.33 (2H, m), 2.38 (2H, t, *J* = 7.4 Hz), 1.78 (2H, m), 1.61 (2H, m), 1.43 (2H, m).

To a solution of the solid (164 mg, 0.54 mmol) obtained above in P(OEt)₃ (852 mg, 5.13 mmol) was added tetrabutylammonium iodide (81.9 mg, 0.22 mmol). The mixture was stirred overnight at reflux temperature. After cooling, the reaction mixture was evaporated in vacuo to remove triethylphosphite. Purification by silica gel flash column chromatography (AcOEt only to AcOEt/MeOH = 9:1) gave 147 mg of **10** as a colorless oil (74%): ¹H NMR (CDCl₃, 500 MHz, δ; ppm) 7.94 (1H, broad s), 7.57 (2H, d, *J* = 7.9 Hz), 7.30 (2H, t, *J* = 7.9 Hz), 7.08 (1H, t, *J* = 7.3 Hz), 6.89 (1H, broad s), 4.13 (4H, m), 3.27 (2H, q, *J* = 6.5 Hz), 2.84 (2H, d, *J* = 20.7 Hz), 2.35 (2H, t, *J* = 7.3 Hz), 1.75 (2H, quintet, *J* = 7.5 Hz), 1.56 (2H, quintet, *J* = 7.3 Hz), 1.42 (2H, quintet, *J* = 7.5 Hz), 1.33 (6H, t, *J* = 7.0 Hz); MS (EI) *m/z*: 384 (M⁺); HRMS Calcd for C₁₈H₂₉N₂O₅P 384.181, Found 384.182.

5.1.19. Thiazolidine-2-carboxylic acid (5-phenylcarbamoylpentyl)amide hydrochloric acid salt (11·HCl). To a solution of thiazolidine-2-carboxylic acid (704 mg, 5.29 mmol) in MeOH (10 mL) was added Boc₂O (1.97 g, 9.04 mmol) and Et₃N (0.5 mL), and the mixture was stirred overnight at room temperature. The solution was concentrated, dissolved with AcOEt and the AcOEt solution was extracted with saturated aqueous NaHCO₃ and the aqueous layer was neutralized with 2 N HCl. The aqueous layer was extracted with ether and the organic layer was washed with brine, and dried over Na₂SO₄. Filtration and concentration in vacuo, and

purification by silica gel flash column chromatography (*n*-hexane/AcOEt = 2:1) gave 966 mg of thiazolidine-2,3-dicarboxylic acid 3-*tert*-butyl ester (78%) as a crude solid: ¹H NMR (CDCl₃, 500 MHz, δ; ppm) 11.09 (1H, broad s), 5.33, 5.15 (1H, s), 3.99, 3.83 (2H, m), 3.24, 3.01 (2H, m), 1.48, 1.45 (9H, s).

2-(5-Phenylcarbamoylpentylcarbamoyl)thiazolidine-3-carboxylic acid *tert*-butyl ester was prepared from amine **27** and thiazolidine-2,3-dicarboxylic acid 3-*tert*-butyl ester obtained above in 79% yield using the procedure described for **25**. ¹H NMR (DMSO-*d*₆, 500 MHz, δ; ppm) 9.85 (1H, s), 7.98 (1H, broad s), 7.58 (2H, d, *J* = 7.9 Hz), 7.28 (2H, t, *J* = 7.9 Hz), 7.01 (1H, t, *J* = 7.5 Hz), 5.14, 5.02 (1H, s), 3.75, 3.67 (2H, m), 3.06 (2H, m), 2.98 (2H, m), 2.29 (2H, t, *J* = 7.7 Hz), 1.58 (2H, quintet, *J* = 7.5 Hz), 1.40 (2H, m), 1.34 (9H, s), 1.32 (2H, quintet, *J* = 7.5 Hz); MS (EI) *m/z*: 421 (M⁺).

To a solution of 2-(5-phenylcarbamoylpentylcarbamoyl)thiazolidine-3-carboxylic acid *tert*-butyl ester (180 mg, 0.43 mmol) in AcOEt (5 mL) was added 4 N HCl·AcOEt (1.0 mL, 4.00 mmol), and the mixture was stirred overnight at room temperature. The solution was concentrated in vacuo and triturated in AcOEt–MeOH–*n*-hexane to give 112 mg (81%) of **11·HCl** as a crude solid. The solid was recrystallized from AcOEt–MeOH and collected by filtration to give 102 mg as colorless crystals: mp 144–147 °C; ¹H NMR (DMSO-*d*₆, 500 MHz, δ; ppm) 9.90 (1H, s), 8.56 (1H, broad s), 7.59 (2H, d, *J* = 7.6 Hz), 7.28 (2H, t, *J* = 7.9 Hz), 7.01 (1H, t, *J* = 7.5 Hz), 5.17 (1H, s), 3.60 (1H, m), 3.45 (1H, m), 3.11 (4H, m), 2.30 (2H, t, *J* = 7.1 Hz), 1.59 (2H, quintet, *J* = 7.5 Hz), 1.47 (2H, quintet, *J* = 7.0 Hz), 1.31 (2H, quintet, *J* = 7.5 Hz); MS (EI) *m/z*: 321 (M⁺); HRMS Calcd for C₁₆H₂₃N₃O₂S 321.151. Found 321.151.

5.1.20. 6-(2-1*H*-Tetrazol-5-ylacetylaminohexanoic acid phenylamide (14). A solution of 1*H*-tetrazole-5-acetic acid (76.8 mg, 0.60 mmol) and **27** (105 mg, 0.51 mmol) in CH₂Cl₂ (1 mL) was cooled to 0 °C and bis(2-oxo-3-oxazolidinyl)phosphinic chloride (206 mg, 0.79 mmol) was added. The mixture was stirred overnight at room temperature and concentrated in vacuo. To the residue was added saturated aqueous NaCl and the precipitate was collected to give 160 mg (99%) of **14** as a pink solid. The solid was recrystallized from MeOH and collected by filtration to give 14 mg of **14** as pink crystals: mp 174–176 °C; ¹H NMR (DMSO-*d*₆, 500 MHz, δ; ppm) 9.88 (1H, s), 8.34 (1H, broad s), 7.58 (2H, d, *J* = 7.6 Hz), 7.28 (2H, t, *J* = 7.9 Hz), 7.01 (1H, t, *J* = 7.5 Hz), 3.86 (2H, s), 3.09 (2H, q, *J* = 6.5 Hz), 2.30 (2H, t, *J* = 7.5 Hz), 1.59 (2H, quintet, *J* = 7.1 Hz), 1.45 (2H, quintet, *J* = 7.5 Hz), 1.31 (2H, quintet, *J* = 7.8 Hz); Anal. Calcd for C₁₅H₂₀N₆O₂·1/4H₂O: C, 56.15; H, 6.44; N, 26.19. Found: C, 56.07; H, 6.25; N, 26.30.

5.1.21. 6-Chloromethanesulfonylamino hexanoic acid phenylamide (19). To a solution of **27** (153 mg, 0.74 mmol) and triethylamine (0.40 mL, 2.87 mmol) in CHCl₃ (3 mL) was added chloromethylsulfonyl chloride (311 mg, 2.09 mmol) dropwise with cooling by an ice-

water bath. The solution was stirred at room temperature for 30 min. The reaction mixture was poured into water and extracted with CHCl₃. The CHCl₃ layer was separated, washed with water, 1 N aqueous HCl and brine, and was dried over Na₂SO₄. Filtration and concentration in vacuo, and purification by silica gel flash column chromatography (*n*-hexane/AcOEt = 2:1 to 1:1) gave 54 mg (23%) of **19** as a yellow solid. The solid was recrystallized from *n*-hexane–CHCl₃ and collected by filtration to give 46 mg of **19** as colorless crystals: mp 122–124 °C; ¹H NMR (CDCl₃, 500 MHz, δ; ppm) 7.51 (2H, d, *J* = 7.9 Hz), 7.32 (2H, t, *J* = 7.9 Hz), 7.15 (1H, broad s), 7.11 (1H, t, *J* = 7.3 Hz), 4.76 (1H, broad s), 4.49 (2H, s), 3.23 (2H, q, *J* = 6.7 Hz), 2.39 (2H, t, *J* = 7.2 Hz), 1.77 (2H, quintet, *J* = 7.5 Hz), 1.65 (2H, quintet, *J* = 7.2 Hz), 1.48 (2H, quintet, *J* = 7.7 Hz); MS (EI) *m/z*: 318 (M⁺); Anal. Calcd for C₁₃H₁₉ClN₂O₃S: C, 48.97; H, 6.01; N, 8.79. Found: C, 48.88; H, 5.85; N, 8.71.

5.1.22. 7-Chlorosulfonylheptanoic acid ethyl ester (29).

To an aqueous solution (7 mL) of anhydrous sodium sulfite (2.03 g, 16.1 mmol) was added a solution of 7-bromoheptanoic acid ethyl ester (**28**, 2.0 g, 8.43 mmol) in EtOH (5 mL) and the solution was boiled under reflux with stirring for 2 h. The solution was evaporated to dryness and the solid was dried in vacuo at 60 °C. This white solid was placed in a flask, toluene (30 mL) was added followed by a catalytic amount of DMF, and then thionyl chloride (6.2 mL, 85.0 mmol) was added dropwise. The mixture was boiled under reflux with stirring for 5 h, diluted with AcOEt, washed with aqueous saturated cold water, and brine, and dried over MgSO₄. Filtration and concentration in vacuo, and purification by silica gel flash chromatography (*n*-hexane/AcOEt = 4:1) gave 2.02 g (93%) of **29**: ¹H NMR (CDCl₃, 400 MHz, δ; ppm) 4.13 (2H, q, *J* = 7.1 Hz), 3.66 (2H, t, *J* = 7.8 Hz), 2.31 (2H, t, *J* = 7.3 Hz), 2.06 (2H, quintet, *J* = 7.8 Hz), 1.66 (2H, quintet, *J* = 7.3 Hz), 1.53 (2H, quintet, *J* = 7.8 Hz), 1.41 (2H, quintet, *J* = 7.1 Hz), 1.26 (2H, quintet, *J* = 7.1 Hz).

5.1.23. 7-Sulfamoylheptanoic acid phenylamide (16). To a mixture of 25% aqueous NH₃ (10 mL), a catalytic amount of 4-(dimethylamino)pyridine, and THF (20 mL) was added a solution of **29** (1.33 g, 5.18 mmol) obtained above in THF (10 mL), and the mixture was stirred at room temperature for 1 h. The reaction mixture was poured into water and extracted with AcOEt. The AcOEt layer was separated, washed with water, saturated aqueous NaHCO₃ and brine, and was dried over Na₂SO₄. Filtration and concentration in vacuo and purification by silica gel flash chromatography (*n*-hexane/AcOEt = 1:1) gave 1.11 g (91%) of 7-sulfamoylheptanoic acid ethyl ester as a crude oil.

Compound **16** was prepared from 7-sulfamoylheptanoic acid ethyl ester obtained above in 64% yield using the procedure described for **4** and **25**: mp 163–164 °C; ¹H NMR (DMSO-*d*₆, 400 MHz, δ; ppm) 9.85 (1H, broad s), 7.58 (2H, d, *J* = 8.1 Hz), 7.28 (2H, t, *J* = 7.6 Hz), 7.01 (1H, t, *J* = 7.1 Hz), 6.72 (2H, broad s), 2.95 (2H, t, *J* = 7.8 Hz), 2.30 (2H, t, *J* = 7.6 Hz), 1.69 (2H, quintet, *J* = 8.0 Hz), 1.59 (2H, quintet, *J* = 7.6 Hz), 1.40 (2H,

quintet, $J = 7.8$ Hz), 1.32 (2H, quintet, $J = 7.1$ Hz); MS (EI) m/z : 284 (M^+); Anal. Calcd for $C_{13}H_{20}N_2O_3S$: C, 54.91; H, 7.09; N, 9.85. Found: C, 54.83; H, 7.18; N, 9.66.

5.1.24. 7-Acetylsulfamoylheptanoic acid phenylamide (7). Compound **7** was prepared from **16** obtained above and acetic acid in 61% yield using the procedure described for **6**: mp 174–176 °C; 1H NMR (DMSO- d_6 , 400 MHz, δ ; ppm) 11.60 (1H, broad s), 9.85 (1H, broad s), 7.58 (2H, d, $J = 7.6$ Hz), 7.28 (2H, t, $J = 7.6$ Hz), 7.01 (1H, t, $J = 7.3$ Hz), 3.34 (2H, t, $J = 7.8$ Hz), 2.29 (2H, t, $J = 7.3$ Hz), 1.65 (2H, quintet, $J = 7.8$ Hz), 1.57 (2H, quintet, $J = 7.3$ Hz), 1.40 (2H, quintet, $J = 7.6$ Hz), 1.30 (2H, quintet, $J = 7.6$ Hz); Anal. Calcd for $C_{15}H_{22}N_2O_4S \cdot 1/10H_2O$: C, 54.89; H, 6.82; N, 8.54. Found: C, 55.03; H, 6.83; N, 8.16.

5.1.25. 7-Bromoheptanoic acid phenylamide (32). 7-Bromoheptanoic acid was prepared from **28** in 99% yield using the procedure described for **4**. In this case, LiOH was used instead of NaOH: 1H NMR ($CDCl_3$, 400 MHz, δ ; ppm) 3.41 (2H, t, $J = 6.8$ Hz), 2.37 (2H, t, $J = 7.3$ Hz), 1.87 (2H, quintet, $J = 6.8$ Hz), 1.66 (2H, quintet, $J = 7.6$ Hz), 1.54–1.32 (4H, m).

To a suspension of 7-bromoheptanoic acid (2.64 g, 12.6 mmol) obtained above in CH_2Cl_2 (30 mL) were added oxalyl chloride (1.65 mL, 18.9 mmol) and a catalytic amount of DMF. The mixture was stirred at room temperature for 2 h. The solvent was removed by evaporation in vacuo to give acid chloride **31**. To a solution of aniline (3.50 g, 37.6 mmol) and triethylamine (5.30 mL, 38.1 mmol) in CH_2Cl_2 (40 mL) was added a solution of **31** obtained above in CH_2Cl_2 (10 mL) dropwise cooling in an ice-water bath. The mixture was stirred at room temperature for 1 h. It was diluted with AcOEt and washed with aqueous saturated $NaHCO_3$, water and brine, before being dried over $MgSO_4$. Filtration and concentration in vacuo, and purification by silica gel flash chromatography (*n*-hexane/AcOEt = 3:1) gave 3.13 g (87%) of **32**: 1H NMR ($CDCl_3$, 400 MHz, δ ; ppm) 7.51 (2H, d, $J = 8.1$ Hz), 7.32 (2H, t, $J = 7.6$ Hz), 7.15 (1H, broad s), 7.10 (1H, t, $J = 7.6$ Hz), 3.41 (2H, t, $J = 6.8$ Hz), 2.36 (2H, t, $J = 7.3$ Hz), 1.87 (2H, quintet, $J = 7.1$ Hz), 1.75 (2H, quintet, $J = 7.3$ Hz), 1.49 (2H, quintet, $J = 7.6$ Hz), 1.41 (2H, quintet, $J = 6.8$ Hz).

5.1.26. 7-Methylsulfanylheptanoic acid phenylamide (33). To a solution of **32** (300 mg, 1.06 mmol) in EtOH (10 mL) was added methylmercaptan sodium salt (15% in water, 1.50 g, 3.21 mmol) and the solution was stirred at room temperature for 5 h. The reaction mixture was diluted with AcOEt and washed with water and brine, and was dried over $MgSO_4$. Filtration and concentration in vacuo, and purification by silica gel flash chromatography (*n*-hexane/AcOEt = 2:1) gave 262 mg (99%) of **33** as a crude solid. The solid was recrystallized from *n*-hexane–AcOEt and collected by filtration to give 217 mg of **33** as a colorless crystal: 1H NMR ($CDCl_3$, 400 MHz, δ ; ppm) 7.51 (2H, d, $J = 8.0$ Hz), 7.32 (2H, t, $J = 7.8$ Hz), 7.16 (1H, broad s), 7.10 (1H, t, $J = 7.6$ Hz), 2.49 (2H, t, $J = 7.1$ Hz), 2.36 (2H, t, $J = 7.3$ Hz), 2.09 (3H, s), 1.74 (2H, quintet, $J = 7.3$ Hz), 1.61

(2H, quintet, $J = 7.3$ Hz), 1.42 (4H, m); MS (EI) m/z : 251 (M^+).

5.1.27. 7-Methanesulfinylheptanoic acid phenylamide (15). To a solution of **33** (80 mg, 0.32 mmol) obtained above in CH_2Cl_2 (3 mL) was added 3-chloroperoxybenzoic acid (65%, 85 mg, 0.32 mmol). The mixture was stirred at room temperature for 30 min. To the reaction mixture was added saturated aqueous $NaHCO_3$ and saturated aqueous $Na_2S_2O_3$, and the mixture was stirred at room temperature for 1 h. The reaction mixture was poured into water and extracted with $CHCl_3$. The $CHCl_3$ layer was separated, washed with water and brine, and was dried over Na_2SO_4 . Filtration and concentration in vacuo and purification by silica gel flash chromatography (AcOEt/MeOH = 10:1) gave 66 mg (77%) of **15** as a crude solid. The solid was recrystallized from *n*-hexane–AcOEt and collected by filtration to give 50 mg of **15** as colorless crystals: mp 121–122 °C; 1H NMR ($CDCl_3$, 400 MHz, δ ; ppm) 7.65 (1H, broad s), 7.54 (2H, d, $J = 7.8$ Hz), 7.31 (2H, t, $J = 7.6$ Hz), 7.09 (1H, t, $J = 7.3$ Hz), 2.80–2.60 (2H, m), 2.56 (3H, s), 2.36 (2H, t, $J = 7.4$ Hz), 1.90–1.65 (4H, m), 1.65–1.35 (4H, m); MS (EI) m/z : 267 (M^+); Anal. Calcd for $C_{14}H_{21}NO_2S$: C, 62.89; H, 7.92; N, 5.35. Found: C, 62.64; H, 7.89; N, 5.35.

5.1.28. 5-(6-Phenylcarbamoylhexylsulfanyl)pentanoic acid ethyl ester (35). To a solution of a mixture of **32** (2.80 g, 9.85 mmol) in EtOH (30 mL) was added potassium thioacetate (1.70 g, 14.9 mmol). The mixture was stirred at reflux temperature for 7 h. The reaction mixture was poured into water and extracted with AcOEt. The AcOEt layer was separated, washed with water, saturated aqueous $NaHCO_3$ and brine, and was dried over Na_2SO_4 . Filtration and concentration in vacuo and purification by silica gel flash chromatography (*n*-hexane/AcOEt = 5:2) gave 2.70 g (98%) of thioacetic acid *S*-(6-phenylcarbamoylhexyl) ester as a crude solid: 1H NMR ($CDCl_3$, 400 MHz, δ ; ppm) 7.51 (2H, d, $J = 8.0$ Hz), 7.32 (2H, t, $J = 7.3$ Hz), 7.22 (1H, broad s), 7.10 (1H, t, $J = 7.3$ Hz), 2.86 (2H, t, $J = 7.1$ Hz), 2.35 (2H, t, $J = 7.3$ Hz), 2.32 (3H, s), 1.73 (2H, quintet, $J = 7.1$ Hz), 1.59 (2H, quintet, $J = 7.1$ Hz), 1.40 (4H, m); MS (EI) m/z : 279 (M^+).

Compound **34** was prepared from **32** obtained above in 87% yield using the procedure described for **4**: 1H NMR ($CDCl_3$, 400 MHz, δ ; ppm) 7.51 (2H, d, $J = 8.0$ Hz), 7.32 (2H, t, $J = 7.6$ Hz), 7.12 (1H, broad s), 7.10 (1H, t, $J = 7.1$ Hz), 2.53 (2H, q, $J = 7.3$ Hz), 2.36 (2H, t, $J = 7.6$ Hz), 1.74 (2H, quintet, $J = 7.1$ Hz), 1.63 (2H, quintet, $J = 7.1$ Hz), 1.42 (4H, m), 1.33 (1H, t, $J = 7.8$ Hz); MS (EI) m/z : 237 (M^+).

To a solution of **34** (1.0 g, 4.21 mmol) obtained above and 5-bromovaleric acid ethyl ester (1.0 mL, 6.32 mmol) in EtOH (15 mL) was added sodium ethoxide in EtOH (20%, 1.60 g, 4.70 mmol) with cooling by an ice-water bath. The solution was stirred overnight at room temperature. The reaction mixture was poured into ice water and extracted with AcOEt. The AcOEt layer was separated, washed with water and brine, and was

dried over Na_2SO_4 . Filtration and concentration in vacuo and purification by silica gel flash chromatography (*n*-hexane/AcOEt = 2:1) gave 1.39 g (91%) of **35** as a white solid: ^1H NMR (CDCl_3 , 500 MHz, δ ; ppm) 7.52 (2H, d, $J = 7.9$ Hz), 7.32 (2H, t, $J = 7.6$ Hz), 7.20 (1H, broad s), 7.10 (1H, t, $J = 7.4$ Hz), 4.13 (2H, q, $J = 7.2$ Hz), 2.53–2.48 (4H, m), 2.36 (2H, t, $J = 7.4$ Hz), 2.32 (2H, t, $J = 7.3$ Hz), 1.76–1.70 (4H, m), 1.63–1.57 (4H, m), 1.50–1.35 (4H, m), 1.25 (3H, t, $J = 7.1$ Hz); MS (EI) m/z : 365 (M^+).

5.1.29. 5-(6-Phenylcarbamoylhexane-1-sulfinyl)pentanoic acid (20). 5-(6-Phenylcarbamoylhexane-1-sulfinyl)pentanoic acid ethyl ester was prepared from **35** obtained above in 81% yield using the procedure described for **15**: ^1H NMR (CDCl_3 , 500 MHz, δ ; ppm) 7.53 (2H, d, $J = 7.9$ Hz), 7.48 (1H, broad s), 7.31 (2H, t, $J = 7.7$ Hz), 7.09 (1H, t, $J = 7.3$ Hz), 4.13 (2H, q, $J = 7.0$ Hz), 2.69 (2H, quintet, $J = 7.1$ Hz), 2.64 (2H, quintet, $J = 6.7$ Hz), 2.38–2.34 (4H, m), 1.83–1.75 (8H, m), 1.60–1.40 (4H, m), 1.26 (3H, t, $J = 7.0$ Hz); MS (EI) m/z : 381 (M^+).

Compound **20** was prepared from 5-(6-phenylcarbamoylhexane-1-sulfinyl)pentanoic acid ethyl ester obtained above in 98% yield using the procedure described for **4**: mp 122–123 °C; ^1H NMR (CDCl_3 , 500 MHz, δ ; ppm) 7.85 (1H, broad s), 7.54 (2H, d, $J = 7.9$ Hz), 7.30 (2H, t, $J = 7.7$ Hz), 7.09 (1H, t, $J = 7.4$ Hz), 2.78 (2H, m), 2.67 (2H, m), 2.39 (2H, t, $J = 6.5$ Hz), 2.36 (2H, t, $J = 7.6$ Hz), 1.88–1.66 (8H, m), 1.58–1.35 (4H, m); MS (EI) m/z : 353 (M^+); Anal. Calcd for $\text{C}_{18}\text{H}_{27}\text{NO}_4\text{S} \cdot 1/5\text{MeOH}$: C, 60.74; H, 7.79; N, 3.89. Found: C, 60.96; H, 7.73; N, 3.52.

5.1.30. 5-(6-Phenylcarbamoylhexane-1-sulfonyl)pentanoic acid (21). To a solution of **35** (600 mg, 1.64 mmol) in CH_2Cl_2 (10 mL) was added 3-chloroperoxybenzoic acid (65%, 960 mg, 3.62 mmol). The mixture was stirred overnight at room temperature. To the reaction mixture was added saturated aqueous NaHCO_3 and saturated aqueous $\text{Na}_2\text{S}_2\text{O}_3$ and the mixture was stirred at room temperature for 1 h. The reaction mixture was poured into water and extracted with CHCl_3 . The CHCl_3 layer was separated, washed with water and brine, and was dried over Na_2SO_4 . Filtration and concentration in vacuo and purification by silica gel flash chromatography (*n*-hexane/AcOEt = 1:3) gave 650 mg (99%) of 5-(6-phenylcarbamoylhexane-1-sulfonyl)pentanoic acid ethyl ester as a crude solid: ^1H NMR (CDCl_3 , 500 MHz, δ ; ppm) 7.52 (2H, d, $J = 8.0$ Hz), 7.32 (2H, t, $J = 7.6$ Hz), 7.28 (1H, broad s), 7.10 (1H, t, $J = 7.6$ Hz), 4.14 (2H, q, $J = 7.0$ Hz), 2.98–2.23 (4H, m), 2.38–2.34 (4H, m), 1.95–1.83 (4H, m), 1.81–1.73 (4H, m), 1.51 (2H, quintet, $J = 7.7$ Hz), 1.45 (2H, quintet, $J = 7$ Hz), 1.26 (3H, t, $J = 7.3$ Hz); MS (EI) m/z : 397 (M^+).

Compound **21** was prepared from 5-(6-phenylcarbamoylhexane-1-sulfonyl)pentanoic acid ethyl ester obtained above in 94% yield using the procedure described for **4**: mp 153–154 °C; ^1H NMR ($\text{DMSO}-d_6$, 500 MHz, δ ; ppm) 12.1 (1H, broad s), 9.85 (1H, s), 7.58 (2H, d, $J = 8.6$ Hz), 7.28 (2H, t, $J = 7.7$ Hz), 7.01

(1H, t, $J = 7.3$ Hz), 3.08 (2H, t, $J = 7.3$ Hz), 3.05 (2H, t, $J = 7.3$ Hz), 2.30 (2H, t, $J = 7.3$ Hz), 2.26 (2H, t, $J = 7.3$ Hz), 1.73–1.52 (8H, m), 1.41 (2H, quintet, $J = 7.4$ Hz), 1.33 (2H, quintet, $J = 7.3$ Hz); MS (EI) m/z : 369 (M^+); Anal. Calcd for $\text{C}_{18}\text{H}_{27}\text{NO}_5\text{S}$: C, 58.51; H, 7.37; N, 3.79. Found: C, 58.43; H, 7.42; N, 3.52.

5.2. Biology

5.2.1. HDAC inhibitory activity assay. The assay of HDAC activity was performed using an HDAC fluorescent activity assay/drug discovery kit (AK-500, BIOMOL Research Laboratories). HeLa nuclear extracts (0.5 μL /well) were incubated at 37 °C with 25 μM of Fluor de Lys™ substrate and various concentrations of samples. Reactions were stopped after 30 min by adding Fluor de Lys™ Developer with trichostatin A, which stops further deacetylation. Then, 15 min after addition of this developer, the fluorescence of the wells was measured on a fluorometric reader with excitation set at 360 nm and emission detection set at 460 nm, and the % inhibition was calculated from the fluorescence readings of inhibited wells relative to those of control wells. The concentration of compound which results in 50% inhibition was determined by plotting the $\log[\text{Inh}]$ versus the logit function of the % inhibition. IC_{50} values of SAHA and compound **15** are determined using a regression analysis of the concentration/inhibition data.

5.2.2. Western blot analysis. HCT116 human colon cancer cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, U.S.A.) and cultured in McCoy5A culture medium containing penicillin and streptomycin, supplemented with fetal bovine serum as described in the ATCC instructions. HCT-116 cells (5×10^5) treated for 8 h with samples at the indicated concentrations in 10% FBS-supplemented McCoy's 5A medium were collected and sonicated. Protein concentrations of the lysates were determined by using a Bradford protein assay kit (Bio-Rad Laboratories); equivalent amounts of proteins from each lysate were resolved in 15% SDS-polyacrylamide gel and then transferred onto nitrocellulose membranes (Bio-Rad Laboratories). After blocking with Tris-buffered saline (TBS) containing 3% skimmed milk for 30 min, the transblotted membrane was incubated with hyperacetylated histone H4 antibody (Upstate Biotechnology) (1:2000 dilution) or acetylated α -tubulin antibody (SIGMA) (1:2000 dilution) in TBS containing 3% skimmed milk at 4 °C overnight. After probed with the primary antibody, the membrane was washed twice with water, followed by incubation with goat anti-rabbit or anti-mouse IgG-horseradish peroxidase conjugates (diluted 1:10000 or 1:5000) for 1.5 h at room temperature and washed twice with water. The immunoblots were visualized by enhanced chemiluminescence.

Acknowledgments

This work was supported in part by a grant from the Health Sciences Foundation from the Ministry of Health, Labor and Welfare of Japan.

References and notes

- (a) Grozinger, C. M.; Schreiber, S. L. *Chem. Biol.* **2002**, *9*, 3; (b) Kouzarides, T. *Curr. Opin. Genet. Dev.* **1999**, *9*, 40; (c) Hassig, C. A.; Schreiber, S. L. *Curr. Opin. Chem. Biol.* **1997**, *1*, 300.
- Taunton, J.; Hassig, C. A.; Schreiber, S. L. *Science* **1996**, *272*, 408.
- Sambucetti, L. C.; Fischer, D. D.; Zabudoff, S.; Kwon, P. O.; Chamberlin, H.; Trogani, N.; Xu, H.; Cohen, D. J. *Biol. Chem.* **1999**, *274*, 34940.
- Leoni, F.; Zaliani, A.; Bertolini, G.; Porro, G.; Pagani, P.; Pozzi, P.; Donà, G.; Fossati, G.; Sozzani, S.; Azam, T.; Bufler, P.; Fantuzzi, G.; Goncharov, I.; Kim, S. H.; Pomerantz, B. J.; Reznikov, L. L.; Siegmund, B.; Dinarello, C. A.; Mascagni, P. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 2995.
- (a) Yoshida, M.; Horinouchi, S.; Beppu, T. *BioEssays* **1995**, *17*, 423; (b) Richon, V. M.; Webb, Y.; Merger, R.; Sheppard, T.; Jursic, B.; Ngo, L.; Civoli, F.; Breslow, R.; Rifkind, R. A.; Marks, P. A. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 5705.
- For a review, see: (a) Miller, T. A.; Witter, D. J.; Belvedere, S. *J. Med. Chem.* **2003**, *46*, 5097; For a review, see: (b) Yoshida, M.; Matsuyama, A.; Komatsu, Y.; Nishino, N. *Curr. Med. Chem.* **2003**, *10*, 2351; For a review, see: (c) Miller, T. A. *Expert Opin. Ther. Pat.* **2004**, *14*, 791.
- Finnin, M. S.; Donigian, J. R.; Cohen, A.; Richon, V. M.; Rifkind, R. A.; Marks, P. A.; Breslow, R.; Pavletich, N. P. *Nature* **1999**, *401*, 188.
- (a) Mulder, G. J.; Meerman, J. H. *Environ. Health Perspect.* **1983**, *49*, 27; (b) Vassiliou, S.; Mucha, A.; Cuniasso, P.; Georgiadis, D.; Lucet-Levannier, K.; Beau, F.; Kannan, R.; Murphy, G.; Knaeuper, V.; Rio, M. C.; Basset, P.; Yiotakis, A.; Dive, V. *J. Med. Chem.* **1999**, *42*, 2610.
- (a) Matsuyama, A.; Yoshimatsu, Y.; Shimazu, T.; Sumida, Y.; Osada, H.; Komatsu, Y.; Nishino, N.; Khochbin, S.; Horinouchi, S.; Yoshida, M. *EMBO J.* **2002**, *21*, 6820; (b) Haggarty, S. J.; Koeller, K. M.; Wong, J. C.; Grozinger, C. M.; Schreiber, S. L. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 4389; (c) Wong, J. C.; Hong, R.; Schreiber, S. L. *J. Am. Chem. Soc.* **2003**, *125*, 5586; (d) Glaser, K. B.; Li, J.; Pease, L. J.; Staver, M. J.; Marcotte, P. A.; Guo, J.; Frey, R. R.; Garland, R. B.; Heyman, H. R.; Wada, C. K.; Vasudevan, A.; Michaelides, M. R.; Davidsen, S. K.; Curtin, M. L. *Biochem. Biophys. Res. Commun.* **2004**, *325*, 683.
- (a) Suzuki, T.; Ando, T.; Tsuchiya, K.; Fukazawa, N.; Saito, A.; Mariko, Y.; Yamashita, T.; Nakanishi, O. *J. Med. Chem.* **1999**, *42*, 3001; (b) Frey, R. R.; Wada, C. K.; Garland, R. B.; Curtin, M. L.; Michaelides, M. R.; Li, J.; Pease, L. J.; Glaser, K. B.; Marcotte, P. A.; Bouska, J. J.; Murphy, S. S.; Davidsen, S. K. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 3443; (c) Wada, C. K.; Frey, R. R.; Ji, Z.; Curtin, M. L.; Garland, R. B.; Holms, J. H.; Li, J.; Pease, L. J.; Guo, J.; Glaser, K. B.; Marcotte, P. A.; Richardson, P. L.; Murphy, S. S.; Bouska, J. J.; Tapang, P.; Magoc, T. J.; Albert, D. H.; Davidsen, S. K.; Michaelides, M. R. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 3331; (d) Vasudevan, A.; Ji, Z.; Frey, R. R.; Wada, C. K.; Steinman, D.; Heyman, H. R.; Guo, Y.; Curtin, M. L.; Guo, J.; Li, J.; Pease, L.; Glaser, K. B.; Marcotte, P. A.; Bouska, J. J.; Davidsen, S. K.; Michaelides, M. R. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 3909; (e) Suzuki, T.; Nagano, Y.; Matsuura, A.; Kohara, A.; Ninomiya, S.; Kohda, K.; Miyata, N. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 4321; (f) Wu, T. Y. H.; Hassig, C.; Wu, Y.; Ding, S.; Schultz, P. G. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 449; (g) Vaisburg, A.; Bernstein, N.; Frechette, S.; Allan, M.; Abou-Khalil, E.; Leit, S.; Moradei, O.; Bouchain, G.; Wang, J.; Woo, S. H.; Fournel, M.; Yan, P. T.; Trachy-Bourget, M.-C.; Kalita, A.; Beaulieu, C.; Li, Z.; MacLeod, A. R.; Besterman, J. M.; Delorme, D. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 283; (h) Suzuki, T.; Kouketsu, A.; Matsuura, A.; Kohara, A.; Ninomiya, S.; Kohda, K.; Miyata, N. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 3313; (i) Suzuki, T.; Matsuura, A.; Kouketsu, A.; Nakagawa, H.; Miyata, N. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 331; (j) Chen, B.; Petukhov, P. A.; Jung, M.; Velená, A.; Eliseeva, E.; Dritschilo, A.; Kozikowski, A. P. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 1389; (k) Suzuki, T.; Nagano, Y.; Kouketsu, A.; Matsuura, A.; Maruyama, S.; Kurotaki, M.; Nakagawa, H.; Miyata, N. *J. Med. Chem.* **2005**, *48*, 1019.
- Mastrolorenzo, A.; Supuran, C. T. *Met.-Based Drugs*. **2000**, *7*, 49.
- Breuer, E.; Salomon, C. J.; Katz, Y.; Chen, W.; Lu, S.; Rösenthaller, G. V.; Hadar, R.; Reich, R. *J. Med. Chem.* **2004**, *47*, 2826.
- Gao, J.; Cheng, X.; Chen, R.; Sigal, G. B.; Bruce, J. E.; Schwartz, B. L.; Hofstadler, S. A.; Anderson, G. A.; Smith, R. D.; Whitesides, G. M. *J. Med. Chem.* **1996**, *39*, 1949.
- Wang, D. F.; Wiest, O.; Helquist, P.; Lan-Hargest, H. Y.; Wiech, N. L. *J. Med. Chem.* **2004**, *47*, 3409.
- (a) Somoza, J. R.; Skene, R. J.; Katz, B. A.; Mol, C.; Ho, J. D.; Jennings, A. J.; Luong, C.; Arvai, A.; Buggy, J. J.; Chi, E.; Tang, J.; Sang, B.-C.; Verner, E.; Wynands, R.; Leahy, E. M.; Dougan, D. R.; Snell, G.; Navre, M.; Knuth, M. W.; Swanson, R. V.; McRee, D. E.; Tari, L. W. *Structure* **2004**, *12*, 1325; (b) Vannini, A.; Volpari, C.; Filocamo, G.; Casavola, E. C.; Brunetti, M.; Renzoni, D.; Chakravarty, P.; Paolini, C.; Francesco, R. D.; Gallinari, P.; Steinkühler, C.; Marco, S. D. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 15064.
- Hubbert, C.; Guardiola, A.; Shao, R.; Kawaguchi, Y.; Ito, A.; Nixon, A.; Yoshida, M.; Wang, X.; Yao, T. *Nature* **2002**, *417*, 455.

Novel Inhibitors of Human Histone Deacetylases: Design, Synthesis, Enzyme Inhibition, and Cancer Cell Growth Inhibition of SAHA-Based Non-hydroxamates

Takayoshi Suzuki,^{*,†} Yuki Nagano,[†] Akiyasu Kouketsu,[†] Azusa Matsuura,[†] Sakiko Maruyama,[‡] Mineko Kurotaki,[‡] Hidehiko Nakagawa,[†] and Naoki Miyata^{*,†}

Graduate School of Pharmaceutical Sciences, Nagoya City University, 3-1 Tanabe-dori, Mizuho-ku, Nagoya, Aichi 467-8603, Japan, and Evaluation Group, Drug Research Department, R. & D. Division, Pharmaceuticals Group, Nippon Kayaku Co., Ltd., 31-12, Shimo 3-chome, Kita-ku, Tokyo 115-8588, Japan

Received October 1, 2004

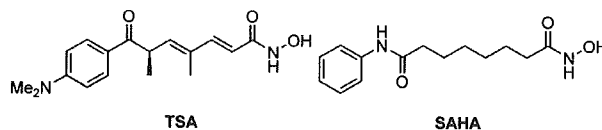
To find novel non-hydroxamate histone deacetylase (HDAC) inhibitors, a series of compounds modeled after suberoylanilide hydroxamic acid (SAHA) was designed and synthesized. In this series, compound **7**, in which the hydroxamic acid of SAHA is replaced by a thiol, was found to be as potent as SAHA, and optimization of this series led to the identification of HDAC inhibitors more potent than SAHA. In cancer cell growth inhibition assay, *S*-isobutryryl derivative **51** showed strong activity, and its potency was comparable to that of SAHA. The cancer cell growth inhibitory activity was verified to be the result of histone hyperacetylation and subsequent induction of p21^{WAF1/CIP1} by Western blot analysis. Kinetic enzyme assay and molecular modeling suggest the thiol formed by enzymatic hydrolysis within the cell interacts with the zinc ion in the active site of HDACs.

Introduction

The reversible acetylation of the ϵ -amino groups of specific histone lysine residues by histone deacetylases (HDACs) and histone acetyl transferases is an important regulatory mechanism of gene expression.¹ When HDACs are inhibited, histone hyperacetylation occurs. The disruption of the chromatin structure by histone hyperacetylation leads to the transcriptional activation of a number of genes.² One important outcome of the activation is induction of the cyclin-dependent kinase inhibitory protein p21^{WAF1/CIP1}, which causes cell cycle arrest.³ Indeed, HDAC inhibitors such as trichostatin A (TSA) and suberoylanilide hydroxamic acid (SAHA) (Chart 1) have been reported to inhibit cell growth, induce terminal differentiation in tumor cells,⁴ and prevent the formation of malignant tumors in mice.⁵ Therefore, HDACs have emerged as attractive targets in anticancer drug development, and HDAC inhibitors have also been viewed as useful tools to study the function of these enzymes.

Many groups have ongoing research programs to find nonpeptide small-molecule inhibitors of HDACs, and these efforts have led to the identification of several classes of inhibitors.⁶ Most previously reported HDAC inhibitors belong to hydroxamic acid derivatives, typified by TSA and SAHA, which are thought to chelate the zinc ion in the active site in a bidentate fashion through its CO and OH groups.⁷ However, hydroxamic acids occasionally have been associated with problems such as poor pharmacokinetics and severe toxicity.⁸ Thus, it has become increasingly desirable to find

Chart 1



replacements that possess strong inhibitory action against HDACs. In addition, in terms of biological research, the discovery of novel zinc-binding groups (ZBGs) may lead to a new type of HDAC isozyme-selective inhibitors which are useful as tools for probing the biology of the enzyme.⁹ Thus far, *o*-aminoanilide,^{9,10} electrophilic ketones,¹¹ and *N*-formyl hydroxylamine¹² have been reported as ZBGs in small-molecule HDAC inhibitors. However, most of them have reduced potency as compared to hydroxamic acid, and unfortunately, HDAC inhibitors bearing electrophilic ketones¹¹ have a metabolic disadvantage in that they are readily reduced to inactive alcohols in vivo, even within cells. We therefore initiated a search for replacement groups for hydroxamic acid with the goal of drug discovery as well as finding new tools for biological research, and found some potent non-hydroxamate small-molecule HDAC inhibitors.¹³ We now present a full account of our study reporting the design, synthesis, HDAC inhibition, cancer cell growth inhibition, and binding mode analysis of non-hydroxamates based on the structure of SAHA.

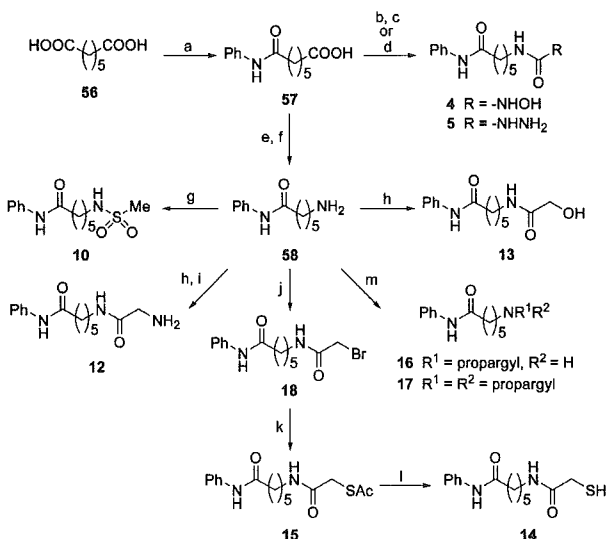
Chemistry

The compounds prepared for this study are shown in Tables 1–5. The routes used for synthesis of the compounds are shown in Schemes 1–4. Scheme 1 shows the preparation of compounds **4**, **5**, **10**, **12–17**, and **18**. Compounds **4** and **5** were synthesized from pimelic acid **56**. The condensation of pimelic acid **56** with an equiva-

* To whom correspondence should be addressed. Tel and fax: +81-52-836-3407; e-mail: miyata-n@phar.nagoya-cu.ac.jp; suzuki@phar.nagoya-cu.ac.jp

[†] Graduate School of Pharmaceutical Sciences, Nagoya City University.

[‡] Nippon Kayaku Co., Ltd.

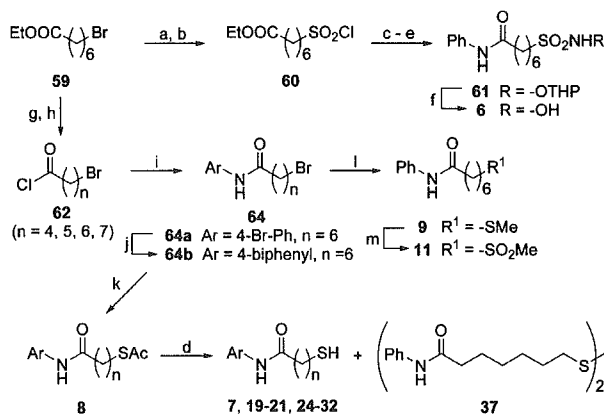
Scheme 1^a

^a Reagents and conditions: (a) aniline, 180 °C; (b) diphenylphosphoryl azide (DPPA), Et₃N, toluene, reflux, and then *O*-(2-tetrahydropyranyl)hydroxylamine, reflux; (c) TsOH, MeOH, rt; (d) DPPA, Et₃N, benzene, reflux, and then hydrazine monohydrate, reflux; (e) DPPA, Et₃N, benzene, reflux, and then BnOH, reflux; (f) H₂, 5% Pd-C, MeOH, rt; (g) MsCl, pyridine, rt; (h) HOCH₂COOH or BocNHCH₂COOH, EDCI, HOBT, DMF, rt; (i) TFA, CHCl₃, rt; (j) BrCH₂COBr, Et₃N, THF, rt; (k) AcSK, EtOH, rt; (l) K₂CO₃, MeOH, rt; (m) propargyl bromide, K₂CO₃, MeOH, rt.

lent amount of aniline gave mono-anilide **57**. Curtius rearrangement of the acyl azide prepared from carboxylic acid **57** using diphenylphosphoryl azide provided the isocyanates, which on treatment with *O*-tetrahydropyranyl (THP) hydroxylamine or hydrazine gave *O*-THP hydroxyurea and semicarbazide **5**. Deprotection of the THP group of the *O*-THP hydroxyurea under acidic conditions afforded hydroxyurea **4**.

Compounds **10**, **12–17**, and **18** were prepared from carboxylic acid **57** obtained above via amine **58** by the procedure outlined in Scheme 1. Carboxylic acid **57** was converted to amine **58** with a three-step sequence: Curtius rearrangement of the acyl azide prepared from carboxylic acid **57**, treatment of the resulting isocyanates with benzyl alcohol, and removal of the Z group by hydrogenation. Coupling between amine **58** and methanesulfonyl chloride afforded sulfonamide **10**. The reaction of amine **58** with *N*-Boc glycine in the presence of EDCI and HOBT in DMF was followed by treatment with trifluoroacetic acid to give aminoacetamide **12**. Hydroxyacetamide **13** was obtained in one step using the procedure described for **12**. The amino group of **58** was acylated with bromoacetyl bromide to yield bromoacetamide **18**. Bromide **18** was treated with potassium thioacetate to give thioacetate **15**, after which deacetylation of the thioacetate in the presence of K₂CO₃ in MeOH gave mercaptoacetamide **14**. The amine **58** was allowed to react with propargyl bromide in the presence of K₂CO₃ to give mono- and di-alkylated compounds **16** and **17**.

Compounds **6–9**, **11**, **19–21**, **24–32**, and **37** were prepared from another starting material, **59** (Scheme 2). The preparation of hydroxysulfonamide **6** was achieved via sulfonyl chloride **60**. Bromide **59** was converted to sulfonyl chloride **60** by sulfation and by a

Scheme 2^a

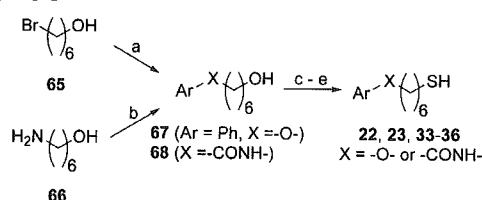
^a Reagents and conditions: (a) Na₂SO₃, EtOH, H₂O, reflux; (b) SOCl₂, DMF, toluene, reflux; (c) *O*-(2-tetrahydropyranyl)hydroxylamine, 4-(dimethylamino)pyridine, pyridine, CH₂Cl₂, rt; (d) 2N aq NaOH, EtOH, rt; (e) aniline, EDCI, HOBT, DMF, rt; (f) TFA, CH₂Cl₂, 60 °C; (g) LiOH·H₂O, EtOH, THF, H₂O, rt; (h) (COCl)₂, DMF, CH₂Cl₂, rt; (i) ArNH₂ (**63**), Et₃N, CH₂Cl₂, rt; (j) PhB(OH)₂, Pd(PPh₃)₄, NaHCO₃, 1-methyl-2-pyrrolidinone, H₂O, 80 °C; (k) AcSK, EtOH, rt; (l) 15% aq NaSMe, EtOH, rt; (m) *m*-chloroperoxybenzoic acid, CH₂Cl₂, rt.

subsequent reaction with thionyl chloride. The sulfonyl chloride **60** was treated with *O*-THP hydroxylamine to give *O*-THP hydroxysulfonamide, after which hydrolysis of the ester under alkaline conditions and subsequent amide formation with aniline gave compound **61**. Removal of the THP group of compound **61** by treatment with trifluoroacetic acid gave hydroxysulfonamide **6**.

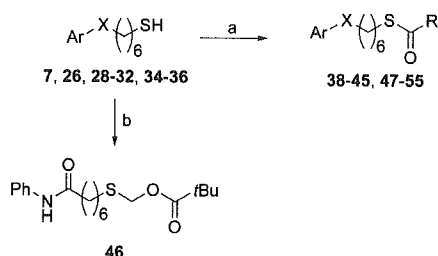
Compounds **7–9**, **11**, **19–21**, **24–32**, and **37** were synthesized from the corresponding acid chlorides **62** (**62a** (*n* = 4) and **62b** (*n* = 5) are commercially available) by the route shown in Scheme 2. **62c** (*n* = 6) was prepared from ester **59** by hydrolysis of the ethyl ester and a subsequent reaction with oxalyl chloride, and **62d** (*n* = 7) was obtained in the same way as **62c**. The amino group of aromatic amines **63** was acylated with an appropriate acid chloride **62** to give the amides **64**. Suzuki coupling¹⁴ of bromobenzene **64a** with phenylboronic acid provided the biphenyl **64b**. Bromides **64** were treated with potassium thioacetate to give compound **8**, after which hydrolysis of the thioacetates under alkaline conditions gave the desired compounds **7**, **19–21**, **24–31**, and **32**, and disulfide **37** was obtained as a byproduct when thiol **7** was synthesized. Sulfide **9** was prepared by the alkylation of methylmercaptan with bromide **64c** (Ar = Ph, *n* = 6). Oxidation of **9** with 2 equiv of *m*-chloroperoxybenzoic acid provided the sulfone **11**.

Thiols **22**, **23**, **33–35**, and **36** were prepared from alcohol **65** or **66** by the procedure outlined in Scheme 3. Treatment of bromide **65** with phenol in the presence of K₂CO₃ gave ether **67**, and condensation of amine **66** with an appropriate aromatic carboxylic acid **69** afforded amides **68**. Alcohols **67** and **68** were converted to thiols **22**, **23**, **33–35**, and **36** in three steps by conversion of the alcohols to bromides, treatment of the bromides with potassium thioacetate, and hydrolysis of the resulting thioacetates.

The preparation of *S*-chemically modified compounds **38–54**, and **55** is shown in Scheme 4. Thiols **7**, **26**, **28–32**, **34**, **35**, and **36** were coupled with the corresponding

Scheme 3^a

^a Reagents and conditions: (a) Phenol, K₂CO₃, DMF, 80 °C; (b) ArCOOH (**69**), EDCI, HOBT, DMF, rt; (c) CBr₄, PPh₃, CH₂Cl₂, 0 °C; (d) AcSK, EtOH, rt; (e) 2N aq NaOH, EtOH, THF, rt.

Scheme 4^a

^a Reagents and conditions: (a) RCOCI (**70**), 4-(dimethylamino)pyridine, pyridine, CH₂Cl₂, rt; (b) NaH, chloromethyl pivalate, DMF, 0 °C to room temperature.

acyl chloride **70** to give thioesters **38–45**, **47–54**, and **55**. Alkylation of thiol **7** with chloromethyl pivalate in the presence of sodium hydride in DMF afforded compound **46**.

Results and Discussion

Enzyme Assays. The compounds synthesized in this study were tested with an in vitro assay using a HeLa nuclear extract rich in HDAC activity. The results are summarized in Tables 1–3.

The IC₅₀ values of SAHA and *o*-aminoanilide **1** were 0.28 μM and 120 μM, respectively (entries 1 and 2). α-Ketoamide **2** and *N*-formyl hydroxylamine **3** were reported previously to inhibit HDACs with an IC₅₀ of 0.34 μM and 2.8 μM, respectively (entries 3 and 4).^{11b,12}

The crystal structures of an archaeobacterial HDAC homologue (HDAC-like protein, HDLP)/hydroxamates and HDAC8/hydroxamates complexes made it clear that the hydroxamic acid group coordinates the zinc ion in the active site through its CO and OH groups and also forms three hydrogen bonds between its CO, NH, and OH groups and Tyr 306, His 143, and His 142 (HDAC8 numbering), respectively.⁷ From these data, hydroxyurea **4**, semicarbazide **5**, and hydroxysulfonamide **6** were synthesized and tested as HDAC inhibitors because it is possible for them to chelate zinc ion and form hydrogen bonds with Tyr and His like SAHA. Among these three compounds, hydroxyurea **4** and semicarbazide **5** showed anti-HDAC activity and the IC₅₀ values were comparable to that of *o*-aminoanilide **1** (entries 5, 6, and 7). However, they were much less effective than SAHA.

Thiols seemed to be reasonable targets for hydroxamic acid replacements, because zinc ion is highly thiophilic and thiol derivatives have been reported to inhibit zinc-dependent enzymes such as angiotensin converting enzyme¹⁵ and matrix metalloproteinases.¹⁶ Furthermore, macrocyclic compounds bearing a disulfide group such as FK228 have been reported recently to inhibit

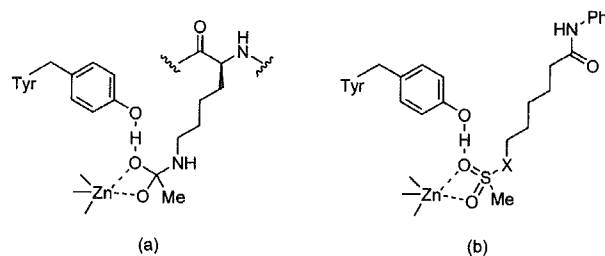


Figure 1. The transition state proposed for HDACs (a), and models for the binding of sulfone derivatives (b).

HDACs under reductive conditions.¹⁷ Surprisingly, although the inhibitory ability of monodentate ZBGs such as thiol was thought to be less than that of bidentate ZBGs such as hydroxamate, *N*-formyl hydroxylamine, and hydrated electrophilic ketones,¹⁶ the activity of thiol **7** was far greater than expected. A pronounced inhibitory effect (IC₅₀ = 0.21 μM) was observed with thiol **7**, which was much more active than previously reported non-hydroxamates such as *o*-aminoanilide, *N*-formyl hydroxylamine, and trifluoromethyl ketone,^{11a} and as potent as α-ketoamide **2** and SAHA (entry 8). To confirm that the thiol group plays an important role in anti-HDAC activity, thioacetate **8a** and methyl sulfide **9** were tested. As expected, thiol transformation into thioacetate and methyl sulfide led to an inhibitor that was about 30-fold less potent and a compound devoid of anti-HDAC activity, respectively (entries 9 and 10). These results suggest that thiolate anion generated under physiological conditions has an intimate involvement in the interaction with the zinc ion in the active site.

The crystal structures of the HDLP/hydroxamates and HDAC8/hydroxamates complexes have led to a solid understanding of not only the three-dimensional structure of the active site of HDACs but also the catalytic mechanism for the deacetylation of acetylated lysine substrate.⁷ It has been proposed that the carbonyl oxygen of this substrate could bind the zinc, and the carbonyl could be attacked by a zinc-chelating water molecule (Figure 2a), which would result in the production of deacetylated lysine via a tetrahedral carbon-containing transition state (Figure 1a). On the basis of the proposed catalytic mechanism, we attempted to design non-hydroxamate HDAC inhibitors. First, we designed transition-state (TS) analogues. The TS of HDAC deacetylation was estimated to include a tetrahedral carbon (Figure 1a) as with other zinc proteases.¹⁸ We focused attention on sulfone derivative TS analogues because it has been suggested that the sulfonamide moiety has strong similarity with the TS of amide bond hydrolysis, both from a steric and an electronic point of view.¹⁹ Compounds **10** and **11**, in which a hydroxamic acid of SAHA is replaced by a sulfonamide and a sulfone, respectively, were designed and synthesized as TS analogues (Figure 1b). Of these two TS analogues, sulfone **11** showed anti-HDAC activity and the IC₅₀ value was 230 μM (entries 11 and 12). However, sulfone **11** was approximately 820-fold less effective than SAHA.

Our next approach was based on the proposed deacetylation mechanism whereby a zinc-chelating water molecule activated by His142 and His 143 (HDAC8 numbering) makes a nucleophilic attack on the carbonyl carbon of an acetylated lysine substrate (Figure 2a).