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ARTICLE

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Electron-transfer mechanism in radical-scavenging reactions by a vitamin E model in a protic medium

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The scavenging reaction of 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$) or galvinoxyl radical (GO$^{\bullet}$) by a vitamin E model, 2,2,5,7,8-pentamethylchroman-6-ol (**1H**), was significantly accelerated by the presence of $Mg(ClO_4)_2$ in de-aerated methanol (MeOH). Such an acceleration indicates that the radical-scavenging reaction of **1H** in MeOH proceeds *via* an electron transfer from **1H** to the radical, followed by a proton transfer, rather than the one-step hydrogen atom transfer which has been observed in acetonitrile (MeCN). A significant negative shift of the one-electron oxidation potential of **1H** in MeOH (0.63 V *vs.* SCE), due to strong solvation as compared to that in MeCN (0.97 V *vs.* SCE), may result in change of the radical-scavenging mechanisms between protic and aprotic media.

Introduction

Recently, much attention has been paid to the mechanisms of radical-scavenging reactions of phenolic antioxidants, such as vitamin E (α-tocopherol) and flavonoids, with regard to the development of chemopreventive agents against oxidative stress and associated diseases. There are two mechanisms for the radical-scavenging reactions of phenolic antioxidants: a one-step hydrogen atom transfer from the phenolic OH group; and an electron transfer followed by a proton transfer.[1-3] Metal ions are a powerful tool that can be used to distinguish between these two mechanisms, since electron-transfer reactions are known to be significantly accelerated by their presence.[4] In fact, we have recently reported that scavenging reactions of the galvinoxyl radical (GO$^{\bullet}$) and the cumylperoxyl radical by (+)-catechin in aprotic media, such as acetonitrile (MeCN) and propionitrile, proceed *via* an electron transfer from (+)-catechin to the radicals (which is significantly accelerated by the presence of metal ions, such as Mg^{2+} and Sc^{3+}) followed by a proton transfer.[5,6] On the other hand, no effect of Mg^{2+} on the hydrogen-transfer rate from a vitamin E model, 2,2,5,7,8-pentamethylchroman-6-ol (**1H**), to 2,2-bis(4-*tert*-octylphenyl)-1-picrylhydrazyl radical (DOPPH$^{\bullet}$) or GO$^{\bullet}$ in de-aerated MeCN has been observed, indicating that the radical-scavenging reactions of **1H** in MeCN proceed *via* a one-step hydrogen atom transfer rather than *via* electron transfer.[7,8] However, the effects of solvents on the mechanism of radical-scavenging reactions of phenolic antioxidants have yet to be clarified. Leopoldini *et al.* have reported that the bond dissociation enthalpies for O–H bonds and the adiabatic ionization potentials for phenolic antioxidants, calculated with use of density functional theory, do not follow the same trends in gas, water and benzene.[2] Thus, it is of considerable importance to investigate the effects of metal ions on radical-scavenging reactions in various solvents with different polarity.[9]

We report herein that the scavenging reactions of 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$) or GO$^{\bullet}$ by the vitamin E model **1H** in de-aerated methanol (MeOH) proceed *via* an electron transfer mechanism rather than *via* a one-step hydrogen atom transfer, which has been observed in de-aerated MeCN. Effects of bases on the radical-scavenging rates were also examined, to clarify whether the actual electron donor is **1H** or the corresponding phenolate anion $\mathbf{1}^-$ in MeOH. Different mechanisms in protic and aprotic solvents are discussed based on kinetic, electrochemical, and EPR data obtained in this study, providing valuable and fundamental information about the radical-scavenging mechanism of phenolic antioxidants.

Experimental

Materials

2,2,5,7,8-Pentamethylchroman-6-ol (**1H**) was purchased from Wako Pure Chemical Ind. Ltd., Japan. 2,2-Diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$) and galvinoxyl radical (GO$^{\bullet}$) were commercially obtained from Aldrich. Tetra-*n*-butylammonium perchlorate (Bu_4NClO_4), used as a supporting electrolyte for the electrochemical measurements, was purchased from Tokyo Chemical Industry Co., Ltd., Japan, recrystallized from ethanol, and dried under vacuum at 313 K. $Mg(ClO_4)_2$ and methanol (MeOH; spectral grade) were purchased from Nacalai Tesque, Inc., Japan and used as received. Pyridine and 2,6-lutidine were commercially obtained from Wako Pure Chemical Ind. Ltd., Japan and purified by the standard procedure.[10]

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Spectral and kinetic measurements

Since the phenoxyl radical of **1H** (**1**•) generated in the reaction of **1H** with radicals readily reacts with molecular oxygen (O_2), reactions were carried out under strictly de-aerated conditions. A continuous flow of Ar gas was bubbled through a MeOH solution (3.0 mL) containing DPPH• (4.8×10^{-5} M) and $Mg(ClO_4)_2$ (0–0.3 M) in a square quartz cuvette (10 mm id) with a glass tube neck for 10 min. Air was prevented from leaking into neck of the cuvette with a rubber septum. Typically, an aliquot of **1H** (2.0×10^{-2} M), which was also in de-aerated MeOH, was added to the cuvette with a microsyringe. This led to a reaction of **1H** with DPPH•. UV-vis spectral changes associated with the reaction were monitored using an Agilent 8453 photodiode array spectrophotometer. The rates of the DPPH•-scavenging reactions of **1H** were determined by monitoring the absorbance change at 516 nm due to DPPH• ($\varepsilon = 1.13 \times 10^4$ M^{-1} cm^{-1}) using a stopped-flow technique on a UNISOKU RSP-1000-02NM spectrophotometer. The pseudo-first-order rate constants (k_{obs}) were determined by a least-squares curve fit using an Apple Macintosh personal computer. The first-order plots of $\ln(A - A_\infty)$ *vs.* time (A and A_∞ are denoted as the absorbance at the reaction time and the final absorbance, respectively) were linear until three or more half-lives with the correlation coefficient $p > 0.999$. The reaction of **1H** with GO• was carried out in the same manner and the rates were determined from the absorbance change at 428 nm due to GO• ($\varepsilon = 1.32 \times 10^5$ M^{-1} cm^{-1}). The rate constants of the reactions in the presence of base (pyridine or 2,6-lutidine) were determined in the same manner.

Electrochemical measurements

The cyclic voltammetry (CV) and second-harmonic alternating current voltammetry (SHACV)[11–16] measurements were performed on an ALS-630A electrochemical analyzer in de-aerated MeOH containing 0.10 M Bu_4NClO_4 as a supporting electrolyte. The Pt working electrode (BAS) was polished with BAS polishing alumina suspension and rinsed with acetone before use. The counter electrode was a platinum wire. The measured potentials were recorded with respect to an $Ag/AgNO_3$ (0.01 M) reference electrode. The $E_{1/2}$ values (*vs.* $Ag/AgNO_3$) were converted to those *vs.* SCE by adding 0.29 V.[17] All electrochemical measurements were carried out at 298 K under 1 atm Ar.

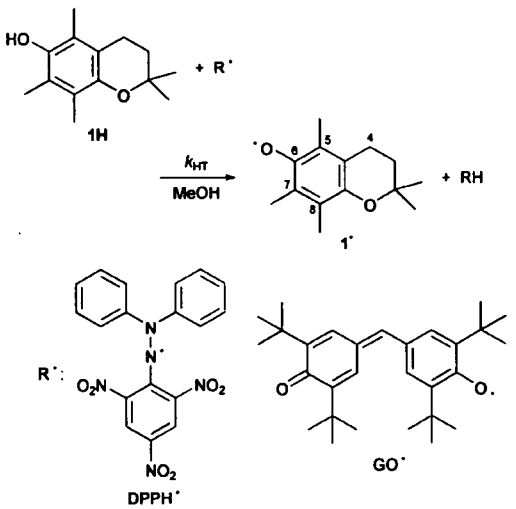
EPR measurements

Typically, an aliquot of a stock solution of **1H** (2.0×10^{-2} M) in de-aerated MeOH was added to the EPR sample tube (0.8 mm id) containing a de-aerated MeOH solution of DPPH• (2.0×10^{-4} M) with a microsyringe under 1 atm Ar. EPR spectra of the phenoxyl radical **1**• produced in the reaction between **1H** and DPPH• were taken on a JEOL X-band spectrometer (JES-RE1XE). The EPR spectra were recorded under non-saturating microwave power conditions. The magnitude of modulation was chosen to optimize the resolution and the signal-to-noise ratio of the observed spectra. The g values and the hyperfine splitting constants were calibrated with a Mn^{2+} marker. Computer simulation of the EPR spectra was carried out using Calleo ESR Version 1.2 program (Calleo Scientific Publisher) on an Apple Macintosh personal computer.

Results and discussion

Radical-scavenging reactions of the vitamin E model in de-aerated MeOH

Upon addition of **1H** to a de-aerated MeOH solution of DPPH•, the absorption band at 516 nm due to DPPH• disappeared immediately, accompanied by an appearance of the absorption band at 427 nm. Since the absorption band at 427 nm is diagnostic of the phenoxyl radical derived from **1H** (**1**•) in MeOH,[18] this spectral change indicates that hydrogen transfer from the phenolic OH group of **1H** to DPPH• takes place to produce **1**• (eqn. (1)). The absorption band of **1**• was shifted from 423 nm in MeCN to 427 nm in MeOH.[7,8] Such a shift in the absorption band of **1**• may be due to a stronger solvation of **1**• in MeOH than in MeCN.



(1)

The rate of the DPPH•-scavenging reaction of **1H** was measured by monitoring the decrease in absorbance at 516 nm due to DPPH• using a stopped-flow technique. The decay of the absorbance at 516 nm due to DPPH• obeyed pseudo-first-order kinetics when the concentration of **1H** ([**1H**]) was maintained at more than a 10-fold excess of the DPPH• concentration. The pseudo-first-order rate constants (k_{obs}) increase with increasing [**1H**], exhibiting first-order dependence on [**1H**]. From the slope of the linear plot of k_{obs} *vs.* [**1H**], the second-order rate constant (k_{HT}) was determined for the radical-scavenging reaction as 1.07×10^3 M^{-1} s^{-1}, in de-aerated MeOH at 298 K. The k_{HT} value thus obtained in de-aerated MeOH is significantly larger than that determined in de-aerated MeCN (4.35×10^2 M^{-1} s^{-1}).[7] A similar result has been reported by Litwinienko and Ingold.[19] Intermolecularly hydrogen-bonded phenolic OH groups of hydrogen-bond accepting solvents, such as alcohols, are known to be essentially unreactive against radicals.[19] Thus, the enhanced k_{HT} value in MeOH suggested that the reaction mechanism in MeOH may be different from that in MeCN. The GO•-scavenging rate constant by **1H** in de-aerated MeOH has also been determined in a same manner by monitoring the decrease in absorbance at 428 nm due to GO• as 2.54×10^3 M^{-1} s^{-1}, which is slightly smaller than that in de-aerated MeCN (3.32×10^3 M^{-1} s^{-1}).

Effect of magnesium ion on the rates of radical scavenging reactions

If the radical-scavenging reactions of **1H** involve an electron-transfer process as the rate-determining step, the rates of radical scavenging would be accelerated by the presence of metal ions.[5,6] This was investigated by examining the effect of $Mg(ClO_4)_2$ on the radical-scavenging rates by **1H** in de-aerated MeOH. When $Mg(ClO_4)_2$ is added to the **1H**–DPPH• system in de-aerated MeOH, the rate of DPPH•-scavenging reaction by **1H** was significantly accelerated. Such an acceleration was not observed for the DPPH•-scavenging reaction by **1H** in MeCN.[7] The k_{HT} value increases linearly with increasing Mg^{2+} concentration ([Mg^{2+}]) as shown in Fig. 1a. A similar acceleration effect of Mg^{2+} has been observed for the GO•-scavenging reaction by **1H** in de-aerated MeOH (Fig. 1b). Thus, the radical-scavenging reactions in de-aerated MeOH may proceed *via* an electron transfer from **1H** to DPPH• or GO•, which is accelerated by the presence of Mg^{2+}, followed by proton transfer from $\mathbf{1H}^{\bullet+}$ to $DPPH^-$ or GO^- as shown in Scheme 1. In such a case,

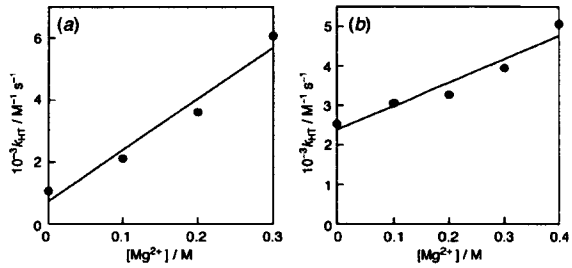
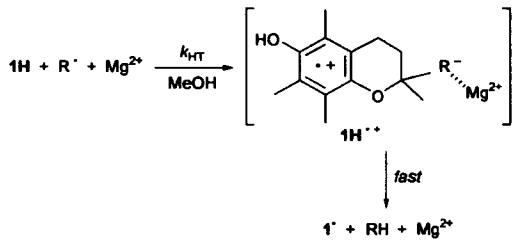


Fig. 1 Plots of k_{HT} *vs.* [Mg^{2+}] in the reaction of **1H** with (a) DPPH$^{\cdot}$ and (b) GO$^{\cdot}$ in de-aerated MeOH at 298 K.



Scheme 1 Radical-scavenging reaction by **1H** *via* an electron transfer in MeOH.

the coordination of Mg^{2+} to $DPPH^-$ or GO^- may stabilize the product, resulting in the acceleration of the electron transfer.

Effect of base on the rates of radical scavenging reactions

In protic media, such as alcohols and water, **1H** may be in equilibrium with the corresponding phenolate anion 1^-, which is a much stronger electron donor as compared to the parent **1H**.[20] In such a case, 1^- may act as an electron donor rather than the parent **1H** in MeOH.

In order to clarify an actual electron donor in MeOH, the effect of base on the radical-scavenging rates of **1H** was examined. The addition of pyridine to the **1H**–DPPH$^{\cdot}$ system results in a significant increase in the rate of the DPPH$^{\cdot}$-scavenging reaction by **1H**. The k_{HT} value increases with increasing pyridine concentration to reach a constant value as shown in Fig. 2. When pyridine is replaced by 2,6-lutidine, a stronger base than pyridine, the limiting k_{HT} value is larger than that in the case of pyridine, as shown in Fig. 2. If the rate of acceleration is due to the deprotonation of the phenolic OH group of **1H** in the presence of base, the limiting k_{HT} value should be the same regardless of the basicity of pyridines. The different limiting k_{HT} values between pyridine and 2,6-lutidine in Fig. 2 suggest that little deprotonation occurs to produce 1^- and that the actual electron donor is the parent **1H** rather than 1^- in MeOH, as shown in Scheme 1. In such a case, the coordination of pyridines

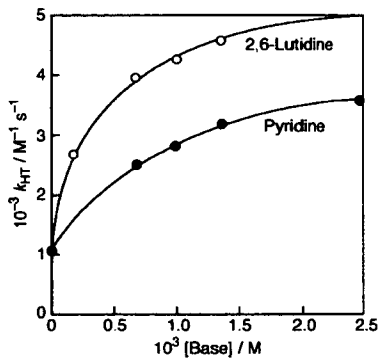


Fig. 2 Plot of k_{HT} *vs.* [base] for the reaction of **1H** with DPPH$^{\cdot}$ in the presence of pyridine (black circles) or 2,6-lutidine (white circles) in de-aerated MeOH at 298 K.

to $1H^{\cdot+}$ may stabilize the product, resulting in the acceleration of the initial electron-transfer process. In the presence of a large amount of a strong Lewis acid, such as $Mg(ClO_4)_2$, no deprotonation of **1H** occurs in MeOH.

Solvent effect on the one-electron oxidation potential of the vitamin E model

The solvent effect on the one-electron oxidation potential (E^0_{ox}) of **1H** was examined by cyclic voltammetry (CV) and second-harmonic alternating current voltammetry (SHACV) measurements.[11–16] Very recently, Williams and Webster have reported that the one-electron oxidation of α-tocopherol itself occurs at about 0.97 V *vs.* SCE in MeCN (0.25 M Bu_4NPF_6) based on the detailed electrochemical analyses.[21] A similar cyclic voltammogram was observed for the electrochemical oxidation of **1H** in MeCN (0.1 M Bu_4NClO_4) (data not shown), from which was determined the E^0_{ox} value (*vs.* SCE) of **1H** in MeCN as 0.97 V. On the other hand, the CV wave of **1H** in MeOH (0.1 M Bu_4NClO_4) was irreversible. Thus, SHACV measurement was carried out to determine the E^0_{ox} value of **1H** in MeOH. The E^0_{ox} value (*vs.* SCE) of **1H** in MeOH (0.1 M Bu_4NClO_4), determined from the intersection of an SHACV wave (Fig. 3), is located at 0.63 V, which is significantly more negative than the value in MeCN (0.97 V). Such a negative shift of the E^0_{ox} value in MeOH as compared to that in MeCN may be ascribed to a stronger solvation of $1H^{\cdot+}$ in MeOH than in MeCN. Thus, the ease of one-electron oxidation of **1H** in MeOH as compared to in MeCN may result in the difference in the radical-scavenging mechanism.

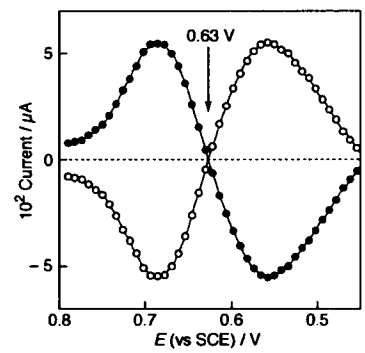


Fig. 3 SHACV of **1H** recorded at the scan rate of 4 mV s^{-1} on Pt working electrode in de-aerated MeOH (0.1 M Bu_4NClO_4) at 298 K.

EPR spectrum of the phenoxyl radical derived from the vitamin E model in de-aerated MeOH

The EPR detection of radical species derived from **1H** would provide valuable information about the solvation of the radical species.[22,23] The EPR spectrum of 1$^{\cdot}$ in de-aerated MeOH at 298 K is shown in Fig. 4a. It should be noted that the *g* value of the EPR spectrum of 1$^{\cdot}$ in MeOH (2.0040) is apparently smaller than that in MeCN (2.0047).[7] The observed hyperfine structure in Fig. 4a is well reproduced by the computer simulation (Fig. 4b) with four hyperfine splitting constants (*hfc*) listed in Table 1. Table 1 also shows the *hfc* values of 1$^{\cdot}$ in MeCN.[7] All the *hfc* values in MeOH are also smaller than those in MeCN. The smaller *g* value of the EPR spectrum of 1$^{\cdot}$ as well as the smaller *hfc* values in MeOH than those in MeCN indicates that the stronger solvation of 1$^{\cdot}$ may occur in MeOH than in MeCN. Although the EPR spectrum of $1H^{\cdot+}$ could not be observed because of the fast deprotonation to produce 1$^{\cdot}$ (Scheme 1), stronger solvation of $1H^{\cdot+}$ may also occur in MeOH than in MeCN, resulting in the ease of one-electron oxidation of **1H** in MeOH than in MeCN.

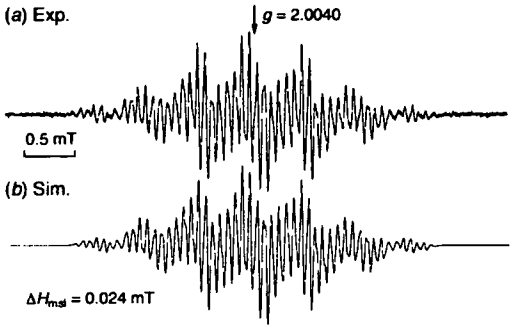


Fig. 4 (a) EPR spectrum of **1**• generated in the reaction of **1H** (1.0×10^{-3} M) with DPPH• (2.0×10^{-4} M) in de-aerated MeOH at 298 K. (b) The computer simulation spectrum. The *hfc* values used for the simulation are listed in Table 1.

Table 1 Hyperfine splitting constants (*hfc*; in mT) and *g* values of **1**• in de-aerated solvents

Solvent	*g*	$a(3H^5)$	$a(3H^7)$	$a(3H^8)$	$a(2H^4)$
MeOH	2.0040	0.577	0.423	0.073	0.126
MeCN	2.0047[a]	0.587[a]	0.440[a]	0.086[a]	0.139[a]

[a] Taken from ref. 7.

In conclusion, the scavenging reaction of DPPH• or GO• by **1H** in MeOH proceeds *via* the electron transfer from **1H** to DPPH• or GO• followed by proton transfer rather than *via* the one-step hydrogen atom transfer, which has been observed in MeCN. Such a difference in the mechanism of radical-scavenging reactions by the vitamin E model depending on the solvents provides valuable information for the biological antioxidative reactions.

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Photoinduced Nitric Oxide Release from Nitrobenzene Derivatives

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Abstract: A new type of photoinduced nitric oxide (NO) donors was designed from nitrobenzene derivatives. Visible-light irradiation of 2,6-dimethylnitrobenzenes bearing extended π-electron systems at the 4-position revealed efficient NO release using ESR analysis and the Griess assay. Computational study and ultraviolet spectrum analysis suggested that the NO-releasing activity was closely related to the conformation of the nitro group, the absorption intensity, 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 4-substituted-2,6-dimethylnitrobenzene analogues are useful NO donors for the time- and site-controlled NO treatment.

Introduction

Nitric oxide (NO), a simple diatomic free radical, has proven in recent years to be involved in the maintenance and regulation of vital functions[1] and is one of the most fascinating and studied compounds in biological chemistry, although for decades it was merely viewed as an environmental pollutant. The development and use of NO donors have played important roles in research on NO physiology,[2] and the significance of these donors has recently been reaffirmed not only from the perspective of reagents for biological studies but also with a view to their application as pharmaceuticals.[3] To date, a number of NO donors have been developed[2,4] and utilized. However, many of the currently used NO donors such as 1-hydroxy-2-oxo-3-(aminoalkyl)-1-triazenes (NOCs)[5] and 4-alkyl-2-hydroxyimino-5-nitro-3-hexenes (NORs)[6] are reagents that release NO by spontaneous autolysis. For further research on NO physiology and potential therapeutic application, it appeared desirable to liberate NO in living systems in a time- and site-controlled manner. This concept led to the identification of several photochemically triggered NO donors such as metal nitrosyl compounds[7] and some caged nitric oxides.[8] The duration and site of NO release from these compounds can be controlled by changing the interval and position of light exposure. However, some of these photoinducible NO donors have problems associated with stability and toxicity. For example, the NO-releasing rate of metal nitrosyl compounds such as dipotassium pentachloronitrosylruthenate and sodium nitroprusside (SNP) varies with changes in pH, and SNP has toxic consequences attributable to the cyanide ligand.[9]

We previously found 6-nitrobenzo[*a*]pyrene (6-nitroBaP) (Figure 1) to be a photoinducible NO-releasing agent[10] whose NO-releasing mechanism is completely different from those of well-known photochemically triggered NO donors. 6-NitroBaP releases NO with the concomitant formation of 6-oxylBaP radical under visible-light irradiation (Figure 1). Based on the fact that 1-nitroBaP and 3-nitroBaP with sterically less hindered nitro

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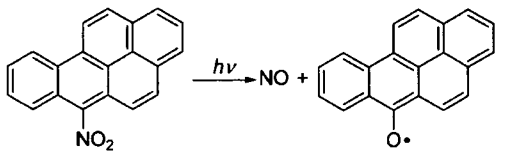
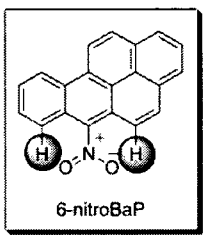
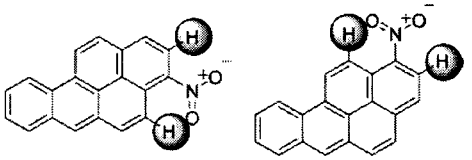


6-nitroBaP

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



release NO



3-nitroBaP 1-nitroBaP

No release of NO

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

groups did not release NO (Figure 2) and the results of computational studies by our group[10] 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 oxylradical 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)−Fe^{2+} complex, which reacts with NO to give a [$(MGD)_2$−Fe^{2+}−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^{iso} = 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 NMe_2 group at the 4-position of 2,6-dimethylnitrobenzene, respectively (data not shown), whereas a 5-min photoirradiation[13] 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 method[14] was used to evaluate production by detecting NO_2^- 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-

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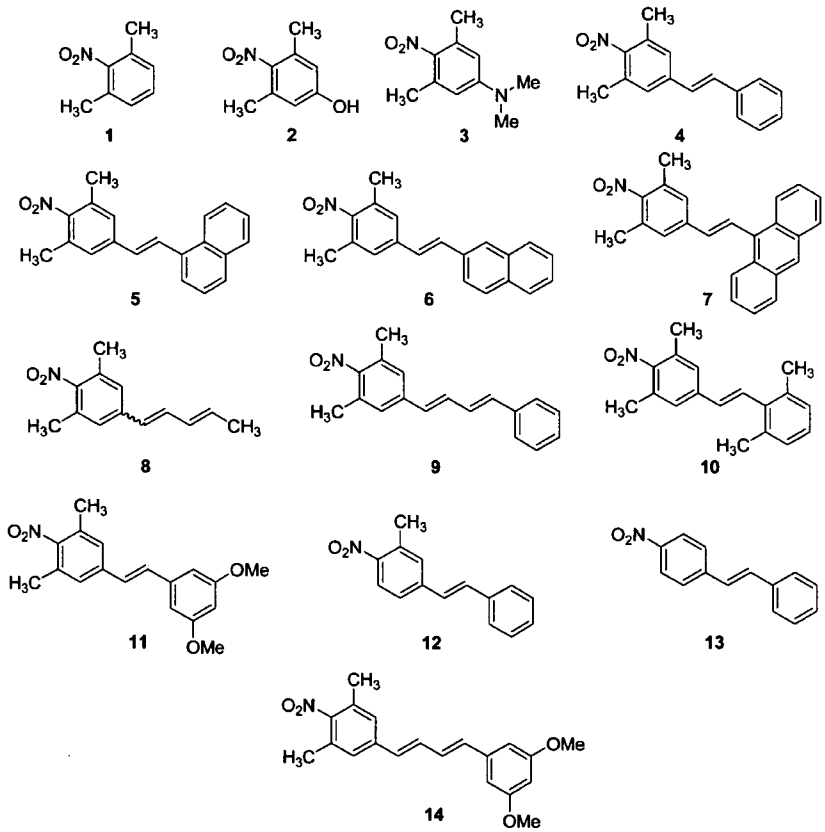

Figure 3. Structures of nitrobenzene derivatives.

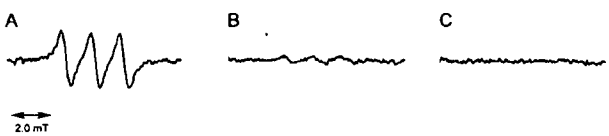

Figure 4. ESR spectra of [(MGD)$_2$–Fe–NO] complex after photoirradiation in the presence of compounds **4**, **12**, and **13**. Samples contained 1 mM **4** (A), **12** (B), or **13** (C), 75 mM MGD, and 20 mM $FeSO_4$ in distilled water (containing 25% DMSO); ESR spectra were recorded after photoirradiation 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

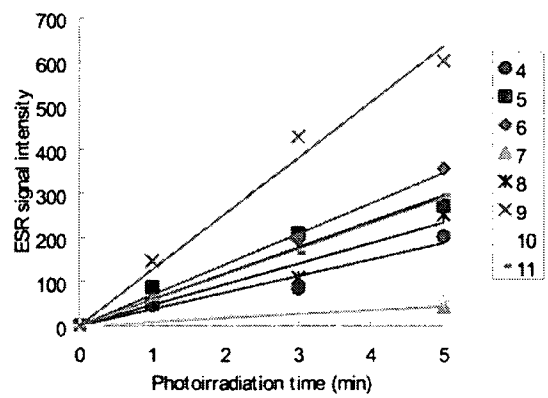

Figure 5. ESR signal intensities of [(MGD)$_2$–Fe–NO] complex after photoirradiation in the presence of compounds **4**–**11**. Samples contained 1 mM **4**–**11**, 75 mM MGD, and 20 mM $FeSO_4$ in water (containing 25% DMSO); ESR spectra were recorded after photoirradiation for 1, 3, or 5 min with a 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

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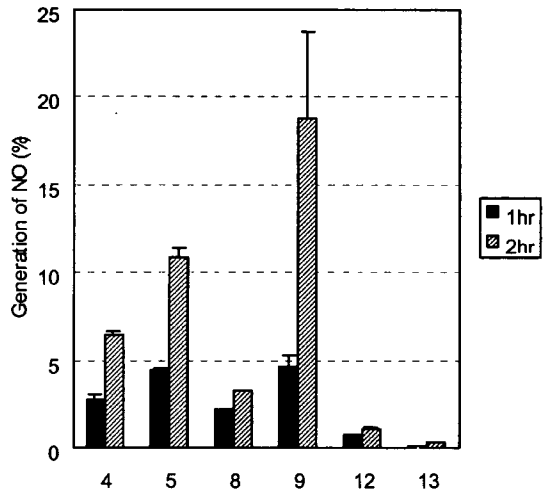


Figure 6. Griess assay results for compounds **4**, **5**, **8**, **9**, **12**, and **13** (n = 3, mean ±SD).

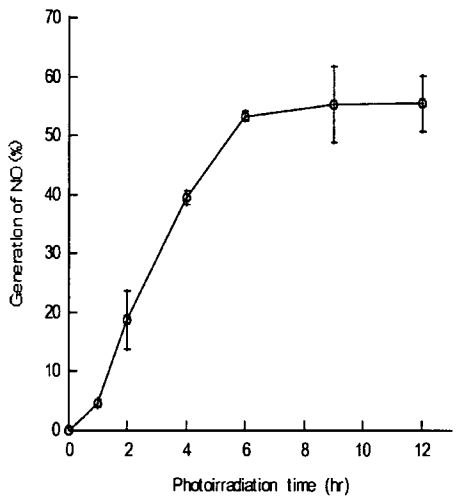


Figure 7. NO-release rate of compound **9** (n = 3, mean ±SD).

was more easily available for the cellular experiments as a DMSO solution. In the presence of a 2.5 μM 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).

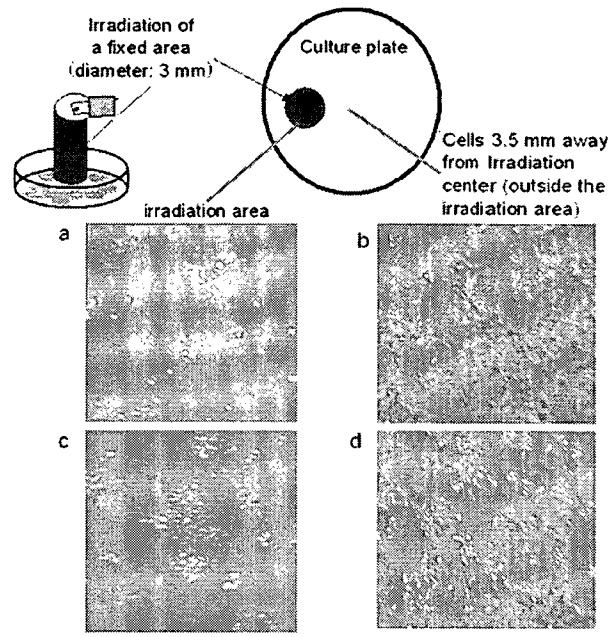


Figure 8. Cytotoxic activity of NO released from compound **14** under photoirradiation. Representative phase-contrast images of cells within the irradiation area in the absence (a) and the presence (c) of P450 nor are shown. Cells at 3.5 mm away from the irradiation center in the absence (b) and the 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.

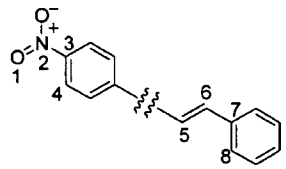
Discussion

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 ($\Delta O^1N^2C^3C^4$) 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



compds	$O^1N^2C^3C^4$[a] (deg)	$C^5C^6C^7C^8$[b] (deg)	λ max (nm)	ε max (× 10^4 M^{-1} cm^{-1})	ESR signal[c] (× 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.

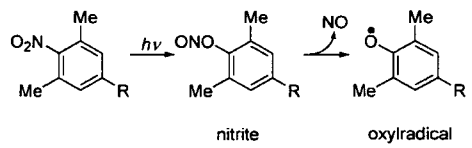


Figure 9. Predicted mechanism for NO generation from 4-substituted-2,6-dimethylnitrobenzene derivatives.

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 oxylradicals generated by N–O bond fission of the nitrite. The destabilization of the oxylradical 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 oxylradical, 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 oxylradical 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 $CDCl_3$ 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 ±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 photoreflector lamp.

3,5-Dimethyl-4-nitrophenol (2). Compound **2** was prepared by a previously reported method:[18] mp 109–111 °C; ^1H NMR ($CDCl_3$, 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 $C_8H_9NO_3$: C, 57.48; H, 5.43; N, 8.38. Found: C, 57.33; H, 5.55; N, 8.61.

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(3,5-Dimethyl-4-nitrophenyl)dimethylamine (3). Compound **3** was prepared by a previously reported method:[19] mp 115−116 °C; ^{1}H NMR ($CDCl_3$, 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 $C_{10}H_{14}N_2O_2$: 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-[(1*E*,3*E*)-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 alcohol[20] (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 $CHCl_3$. The $CHCl_3$ layer was separated, washed with brine, and dried over Na_2SO_4. 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; ^{1}H NMR ($CDCl_3$, 400 MHz, δ; ppm) 7.15 (2H, s), 4.40 (2H, s), 2.31 (6H, s). MS (EI) *m/z*: 243 (M^+, ^{79}Br), 245 (M^+, ^{81}Br).

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 ($CHCl_3$/MeOH = 100/1) to give 873 mg (q.y.) of 3,5-dimethyl-4-nitrobenzylphosphonate as a pale yellow oil: ^{1}H NMR ($CDCl_3$, 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 $C_{13}H_{20}NO_5P$, 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 $CHCl_3$. The $CHCl_3$ layer was separated, washed with brine, and dried over Na_2SO_4. 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; ^{1}H NMR ($CDCl_3$, 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 Na_2SO_4. 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; ^{1}H NMR ($CDCl_3$, 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-[(1*E*,3*E*)-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; ^{1}H NMR ($CDCl_3$, 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 $C_{19}H_{19}NO_3$: 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; ^{1}H NMR ($CDCl_3$, 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 $C_{16}H_{15}NO_2$: 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; ^{1}H NMR ($CDCl_3$, 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 $C_{20}H_{17}NO_2$: 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; ^{1}H NMR ($CDCl_3$, 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 $C_{20}H_{17}NO_2$: 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; ^{1}H NMR ($CDCl_3$, 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 $C_{24}H_{19}NO_2$: 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; ^{1}H NMR ($CDCl_3$, 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 $C_{13}H_{15}NO_2$, 217.110; found, 217.110.

1,3-Dimethyl-2-nitro-5-[(1*E*,3*E*)-4-phenyl-1,3-butadienyl]benzene (9): mp 126−130 °C; ^{1}H NMR ($CDCl_3$, 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 $C_{18}H_{17}NO_2$: 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; ^{1}H NMR ($CDCl_3$, 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 $C_{20}H_{17}NO_2$: 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; ^{1}H NMR ($CDCl_3$, 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 $C_{18}H_{19}NO_4$: 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; ^{1}H NMR ($CDCl_3$, 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 $C_{15}H_{13}NO_2$: 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; ^{1}H NMR ($CDCl_3$, 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 $C_{14}H_{11}NO_2$: C, 74.65; H, 4.92; N, 6.22. Found: C, 74.65; H, 4.92; N, 6.22.

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ESR Analysis. The Fe^{2+} complex of MGD [Fe^{2+}−MGD_2, (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 curette 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.

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Design and synthesis of non-hydroxamate histone deacetylase inhibitors: identification of a selective histone acetylating agent

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Abstract—A series of suberoylanilide hydroxamic acid (SAHA)-based non-hydroxamates was designed, synthesized, and evaluated for their histone deacetylase (HDAC) inhibitory activity. Among these, methyl sulfoxide **15** inhibited HDACs in enzyme assays and caused hyperacetylation of histone H4 while not inducing the accumulation of acetylated α-tubulin in HCT116 cells.

1. Introduction

The dynamic homeostasis of the nuclear acetylation of histones is regulated by the opposing activity of the enzymes, histone acetyl transferases (HATs) and histone deacetylases (HDACs). The reversible acetylation of histone lysine residues by HATs and HDACs is one of the several possible regulatory mechanisms 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 genes[2] associated with some disease states such as cancer[3] and inflammation.[4] Indeed, HDAC inhibitors such as trichostatin A (TSA) and suberoylanilide hydroxamic acid (SAHA) (Fig. 1) have potent anti-cancer effects and some of them are currently in phase I/II clinical trials.[5]

Previously reported HDAC inhibitors are mostly hydroxamic acid derivatives,[6] 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 produced problems such as poor pharmacokinetics and severe toxicity.[8] In addition, although isozyme-selective HDAC inhibitors are considered to be useful not only as tools for probing the biology of the enzyme but also as drugs with low toxicity, many of the known hydroxamate HDAC inhibitors do not distinguish well among the HDAC isozymes.[9] Therefore, it is desirable to find non-hydroxamate HDAC inhibitors to improve the pharmacokinetics, toxicity, and isozyme selectivity of hydroxamates. To date, some types of non-peptide non-hydroxamate HDAC inhibitors have been reported.[10] However, many of these have either reduced potency or metabolic disadvantages. Thus, there remains a need to develop non-hydroxamate HDAC inhibitors. Here, we report the design, synthesis, enzyme inhibition, and in-cell selective histone deacetylase inhibition of SAHA-based non-hydroxamates.

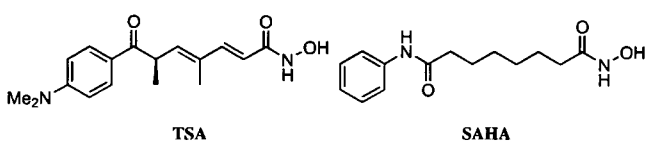

Figure 1. Structures of TSA and SAHA.

Keywords: Histone deacetylase inhibitor; Non-hydroxamate.
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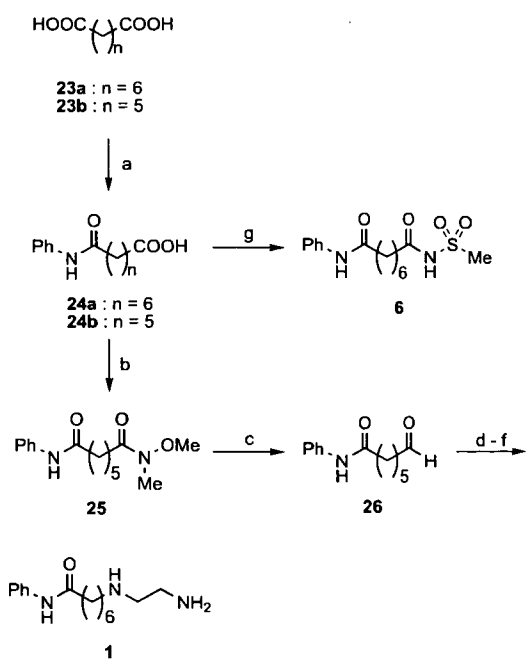
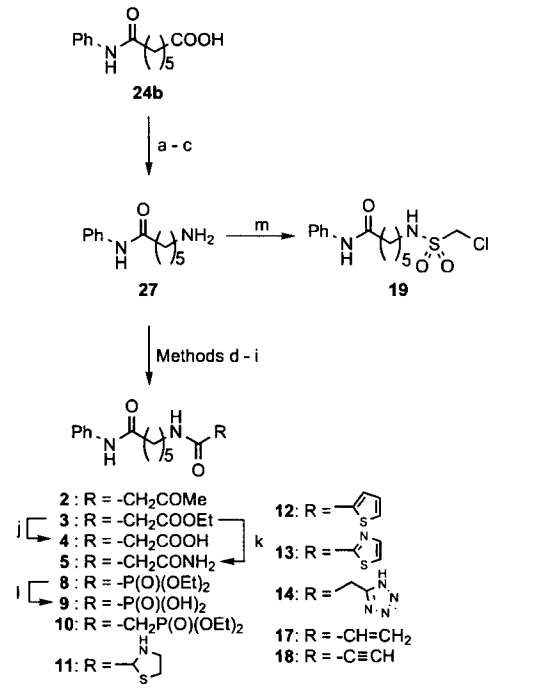
2. Chemistry

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 dicarboxylic acids **23** with an equivalent amount 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.

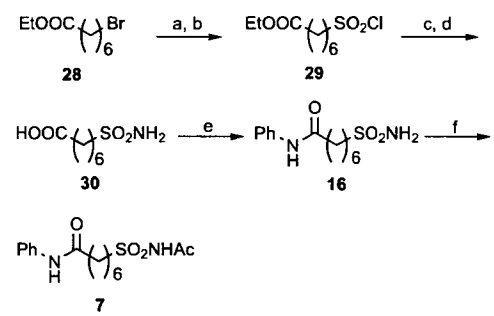
Amine **27** served as a key common intermediate for the synthesis of compounds **2–5**, **8–14**, **17**, and **18** (Scheme 2). Carboxylic acid **24b** was converted to amine **27** by Curtius rearrangement of carboxylic acid **24b**, treatment of the resulting isocyanates with benzyl alcohol, and 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,O*-dimethylhydroxylamine hydrochloride, Et_3N, EDCI, HOBt, DMF, rt; (c) $LiAlH_4$, THF, 0 °C; (d) *N*-(2-aminoethyl)carbamic acid *tert*-butyl ester, $NaBH(OAc)_3$, CH_2Cl_2, AcOH, rt; (e) $(Boc)_2O$, Et_3N, rt; (f) TFA, CH_2Cl_2, rt; (g) $MsNH_2$, 1,1′-carbonyldiimidazole, DBU, DMF, rt.

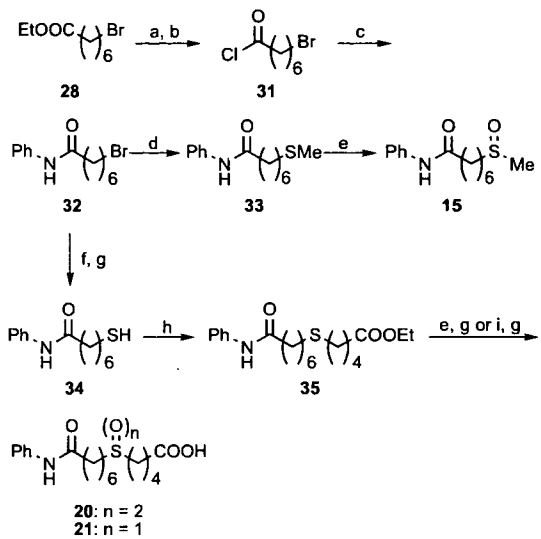


Scheme 2. Reagents and conditions: (a) diphenylphosphoryl azide, Et_3N, benzene, reflux; (b) BnOH, reflux; (c) H_2, 5% Pd–C, MeOH; (d) diketene, rt (for **2**); (e) RCOOH, EDCI, HOBt, DMF, rt (for **3**, **12**, **13**, **17**, and **18**); (f) $EtSCOP(O)(OEt)_2$, rt (for **8**); (g) (i) bromoacetyl chloride, Et_3N, 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 NH_3, MeOH, rt; (l) TMSBr, MeCN, rt; (m) chloromethylsulfonyl chloride, Et_3N, 0 °C to rt.



Scheme 3. Reagents and conditions: (a) Na_2SO_3, EtOH, H_2O, reflux; (b) $SOCl_2$, DMF, toluene, reflux; (c) 25% aq NH_3, 4-(dimethylamino)pyridine, pyridine, CH_2Cl_2, 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 diethylphosphonothiolformate, and following ester group removal with trimethylsilyl bromide gave carbamoylphosphonic acid **9**. Acylation of amine **27** with bromoacetyl chloride followed by an Arbazov reaction provided diethyl phosphonate **10**. Coupling between amine **27** and *N*-Boc



Scheme 4. Reagents and conditions: (a) $LiOH{\cdot}H_2O$, EtOH, THF, H_2O, rt; (b) $(COCl)_2$, DMF, CH_2Cl_2, rt; (c) aniline, Et_3N, CH_2Cl_2, rt; (d) 15% aq NaSMe, EtOH, rt; (e) 1 equiv of *m*-chloroperoxybenzoic acid, CH_2Cl_2, 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, CH_2Cl_2, 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 Na_2SO_3 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**.

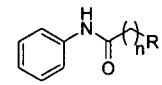
Compounds **15**, **20**, and **21** were synthesized from ester **28** as depicted in Scheme 4. Acid chloride **31** was prepared from ester **28** by hydrolysis of the ethyl ester and subsequent reaction with oxalyl chloride. The amino group of aniline was acylated with the acid chloride **31** to 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**.

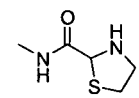
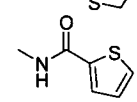
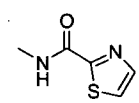
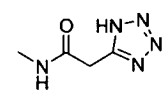
3. Results and discussion

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 IC_{50} of 0.28 μM.

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



Compound	R	*n*	% inhibition at 100 μM
1	$-NHCH_2CH_2NH_2$	6	12 ± 1.2
2	$-NHCOCH_2COMe$	5	12 ± 1.3
3	$-NHCOCH_2COOEt$	5	0 ± 1.4
4	$-NHCOCH_2COOH$	5	4.1 ± 0.56
5	$-NHCOCH_2CONH_2$	5	1.1 ± 2.2
6	$-CONHSO_2Me$	6	21 ± 3.0
7	$-SO_2NHAc$	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	$-NHCOCH_2P(O)(OEt)_2$	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	$-SO_2NH_2$	6	10 ± 0.97
17	$-NHCOCH{=}CH_2$	5	11 ± 3.5
18	$-NHCOC{\equiv}CH$	5	3.7 ± 2.3
19	$-NHSO_2CH_2Cl$	5	0 ± 0.56
20	$-SO_2(CH_2)_4COOH$	6	6.1 ± 3.3
21	$-S(O)(CH_2)_4COOH$	6	12 ± 6.1
22	$-SO_2Me$	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 archaebacterial 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 SO_2 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 sulfonamide[13] (**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,

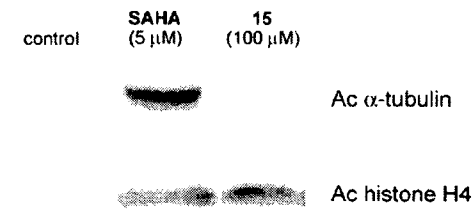


Figure 2. Western blot analysis of histone hyperacetylation and α-tubulin acetylation induction in HCT 116 cells produced by compound **15** and by reference compound SAHA.

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 (^{1}H 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 ±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 $NaHCO_3$ 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: ^{1}H 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 $NaHCO_3$ and brine, and was dried over Na_2SO_4. 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: ^{1}H NMR ($CDCl_3$, 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: ^{1}H NMR ($CDCl_3$, 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 ditrifluoroacedic acid salt (1·2TFA). To a solution of **26** (240 mg, 1.09 mmol) obtained above in CH_2Cl_2 (3 mL) and AcOH (1 mL) was added *N*-(2-aminoethyl)carbamic 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 $NaHCO_3$ 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 120 mg of colorless oil. To a solution of the oil in CH_2Cl_2 (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; ^{1}H 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 $C_{15}H_{25}N_3O$·2TFA: 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**: ^{1}H 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; ^{1}H 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)carbamic 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)carbamic 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-Oxobutyrylamino)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 $C_{16}H_{19}N_3O_2$ S 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; ^{1}H 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 $C_{15}H_{20}N_2O_4$: 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 NH_3 (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; ^{1}H 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 $C_{15}H_{21}N_3O_3$: 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 diethylphosphonothiolformate as a crude oil: ^{1}H NMR ($CDCl_3$, 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 diethylphosphonothiolformate (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: ^{1}H NMR ($CDCl_3$, 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 $C_{17}H_{27}N_2O_5P$ 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; ^{1}H 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 $C_{13}H_{19}N_2O_5P$: 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), Et_3N (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 $CHCl_3$. The solution was washed with saturated aqueous $NaHCO_3$, water, and brine, and was dried over Na_2SO_4. 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: ^{1}H NMR ($CDCl_3$, 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%): ^{1}H NMR ($CDCl_3$, 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 $C_{18}H_{29}N_2O_5P$ 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 Boc_2O (1.97 g, 9.04 mmol) and Et_3N (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 $NaHCO_3$ 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 Na_2SO_4. 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: ^{1}H NMR ($CDCl_3$, 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**. ^{1}H 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; ^{1}H 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 $C_{16}H_{23}N_3O_2S$ 321.151. Found 321.151.

5.1.20. 6-(2-1*H*-Tetrazol-5-ylacetylamino)hexanoic 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 CH_2Cl_2 (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; ^{1}H 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 $C_{15}H_{20}N_6O_2 \cdot 1/4H_2O$: C, 56.15; H, 6.44; N, 26.19. Found: C, 56.07; H, 6.25; N, 26.30.

5.1.21. 6-Chloromethanesulfonylaminohexanoic acid phenylamide (19). To a solution of **27** (153 mg, 0.74 mmol) and triethylamine (0.40 mL, 2.87 mmol) in $CHCl_3$ (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 $CHCl_3$. The $CHCl_3$ layer was separated, washed with water, 1 N aqueous HCl and brine, and was dried over Na_2SO_4. 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–$CHCl_3$ and collected by filtration to give 46 mg of **19** as colorless crystals: mp 122–124 °C; ^{1}H NMR ($CDCl_3$, 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 $C_{13}H_{19}ClN_2O_3S$: 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 $MgSO_4$. Filtration and concentration in vacuo, and purification by silica gel flash chromatography (*n*-hexane/AcOEt = 4:1) gave 2.02 g (93%) of **29**: ^{1}H NMR ($CDCl_3$, 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 NH_3 (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 $NaHCO_3$ and brine, and was dried over Na_2SO_4. Filtration and concentration in vacuo and purification by silica gel flash chromatography (*n*-hexane/AcOEt = 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; ^{1}H 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,