New Strategy for Antedrug Application: Development of Metalloproteinase Inhibitors as Antipsoriatic Drugs

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Phosphonamide-based inhibitors were synthesized and evaluated for the inhibitory activities against the shedding of epidermal growth factors, amphiregulin and heparin-binding EGF-like growth factor, that would participate in the development of psoriasis. All compounds exhibited excellent inhibitory activities for these EGF sheddings; however, they also inhibited matrix metalloproteinases (MMPs). To avoid adverse effects reported by the clinical development of MMP inhibitors, the antedrug concept was introduced. Among the phosphonamide inhibitors, the 2,2,2-trifluoroethyl ester **8d** and 2,2-difluoroethyl ester **8c** showed rapid decomposition in human plasma, which is an essential property for the antedrug. Topical applications of these compounds significantly suppressed TPA-induced epidermal hyperplasia in murin skin, a model of psoriasis. These results suggested that the phosphonamide-based inhibitors have a therapeutic potential for the treatment of psoriasis as an antedrug application.

Introduction

Psoriasis is an inflammatory skin disease and affects 1−3% of the world's population. The feature of the disease is epidermal hyperproliferation of epidermal keratinocytes.[1−3] Several systemic and topical therapies are available, such as methotrexate, retinoids, corticosteroid, vitamin D_3 analogue, and tazarotene, but the effects of these agents are not sufficient.[2] In addition, undesirable adverse effects limit the use of these agents for the therapy. Although the etiology of psoriasis has not been clearly elucidated, aberrant expression of growth factors for epidermal keratinocyte, such as amphiregulin (AR)[4] and heparin-binding EGF-like growth factor (HB-EGF),[5] would participate in the development of psoriatic inflammation.[3,6−8] These epidermal growth factors (EGFs) are synthesized as membrane-anchored precursors, which are converted to the soluble growth factors.[8] Several studies have suggested that zinc-dependent metalloproteases (MPs) are involved in the processing of these membrane-anchored precursors;[9−12] however, the responsible enzymes have not been isolated yet. Therefore, inhibitors of such EGF production in the epidermis are considered to be effective agents for the treatment of psoriasis.

We have already reported that a series of phosphonamide-based inhibitors (**1**) exhibited potent inhibition against HB-EGF shedding ($IC_{50} < 1\ \mu M$).[13] Moreover, these inhibitors also have potent inhibitory activities against matrix metalloproteinases (MMPs). However, it was thought that the inhibition of MMPs may cause undesirable side effects.

In fact, it has been reported that adverse effects such as musculoskeletal syndrome were observed in the clinical studies of MMP broad inhibitors.[14] To overcome such side effects, the antedrug concept was introduced. The term "antedrug" was first defined by Lee et al. in 1982 as locally effective drugs that topically showed activity at the site of application but rapidly metabolized to inactive compounds upon entry into the systemic circulation[15] and thus resulted in no systemic toxicity. This antedrug concept has been mostly applied to antiinflammatory glucocorticoid derivatives to reduce their side effects.[16−19] We expected that the phosphonamide derivatives **1** would be easily metabolized to inactive compounds in blood because the P−OR ester bond in **1** was expected to be easily hydrolyzed by nonspecific plasma esterases (Scheme 1).[20,21] Then, it was expected that the resulting phosphonic acid **2** would be spontaneously decomposed to inactive compounds **3** and **4**.[22]

In this paper, we describe the synthesis and evaluation of the phosphonamide-based derivatives as EGF shedding inhibitors. These compounds were also tested for the stability in human plasma to evaluate the potential of the antedrug. In addition, the degradation mechanism in plasma was investigated by using ^{31}P NMR. Finally, the selected compounds were subjected to in vivo assay using 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced inflammation and hyperplasia in murine skin, a model of psoriasis.

Chemistry

The syntheses for the phosphonamide derivatives were shown in Scheme 2. Commercially available *p*-methoxyphenylphosphonic dichloride **5** was treated with (3*R*)-1,2,3,4-tetrahydroisoquinoline derivative **6** in py-

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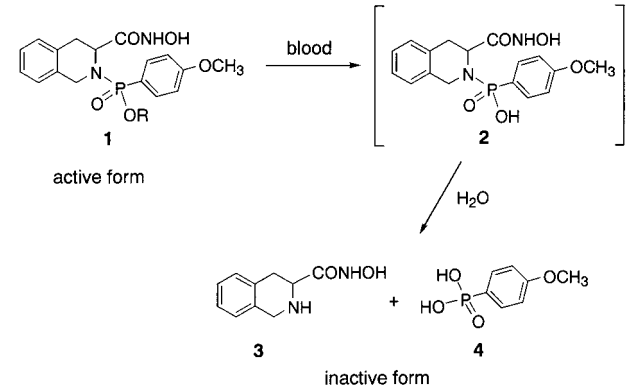
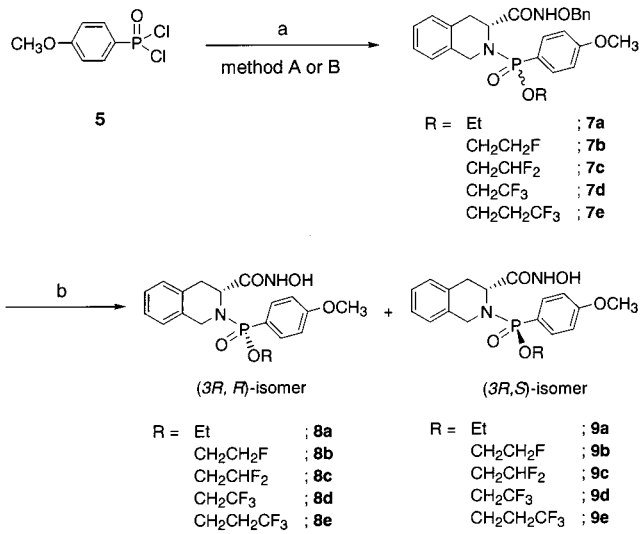
Scheme 1



Scheme 2[a]



[a] (a) Method A: (1) (3*R*)-*N*-benyloxy-1,2,3,4-tetrahydroisoquinoline-3-hydroxamide (**6**), then ROH, pyridine. Method B: (1) ROH (2.1 equiv), NaH, THF; (2) KOH, then $SOCl_2$, catalyst DMF; (3) **6**, diisopropylethylamine, THF. (b) H_2, Pd−C.

ridine, and then the resulting monochloride was allowed to react with the appropriate alcohol to give the phosphonamide **7** (ca. 2:1 diastereomeric mixture) in a one-pot reaction (method A). Alternatively, *p*-methoxyphenylphosphonic dichloride **5** was treated with the appropriate sodium alkoxide to provide the corresponding diester and then selectively hydrolyzed by potassium hydroxide to give the corresponding monoester, which was converted to the corresponding phosphonyl chloride during reflux with thionyl chloride in the presence of catalytic DMF. The resulting phosphonyl chloride was then coupled with (3*R*)-1,2,3,4-tetrahydroisoquinoline derivative **6** in THF in the presence of diisopropylethylamine (DIEA) to yield the phosphonamide **7** as a mixture of the 1:1 diastereomer (method B). Deprotection of the benzyl group in **7** with 10% Pd−C gave the diastereomerically pure hydroxamic acids **8** and **9** that were successfully separated by HPLC purification. Compound **9a** was crystallized and analyzed by X-ray diffraction analysis,[23] and the stereochemistry at the phosphorus atom of compound **9a** was determined to be *S* configuration.[13] The stereochemistries of the other compounds were assigned by a characteristic signal pattern in ^{1}H NMR, especially in an aromatic region. All compounds synthesized here were found to be stable under assay conditions. However, the decompositions of the compounds were observed under acidic conditions (below pH 4) because of the lability of the P−N bond as previously described.[13]

Results and Discussion

At first, we synthesized and evaluated the ethyl ester derivative as a typical phosphonamide inhibitor. The previous work in our laboratory has demonstrated that the stereochemistries at the C-3 and the phosphorus atom of the phosphonamide inhibitors were very important for the inhibitory activities against MPs.[13] Namely, the compounds having (*R*,*R*)-configuration only showed potent inhibitory activities, as shown in Table 1 (compounds **8a** and **9a**). We therefore subjected only (*R*,*R*)-isomers in the series to further investigations.

Initially, we tested the stability of the ethyl ester **8a** in human plasma for 60 min at 37 °C (Table 1). The result was expressed as a percentage of the test compound remaining. Contrary to our expectation, no decomposition was observed in plasma after incubation for 1 h. Then, we designed and synthesized compounds **8b**−**8e**, having various ester groups, to find unstable compounds in plasma. Generally, a good leaving group would facilitate not only chemical hydrolysis but also enzymatic hydrolysis. Thus, fluorine atoms were introduced into the ester group as electron-withdrawing functions to decrease the stability of the ester bond (compounds **8b**−**8e**). All compounds synthesized here were essentially stable in an aqueous solution at pH 4−9 and also under inhibitory assay conditions. As shown in Table 1, all compounds exhibited potent inhibitory activities against HB-EGF and AR shedding (compounds **8b**−**8e**). These compounds also showed potent inhibition against MMP-1, -3, and -9 with nanomolar K_i values. These compounds were then subjected to the stability test in plasma. As shown in Table 1, it was found that the trifluoroethyl ester **8d** was extremely rapidly decomposed in plasma within 1 min. Interestingly, the difluoroethyl ester **8c** showed a moderate decomposition rate (half-life in plasma, ca. 10 min) compared with **8d**. The monofluoroethyl ester **8b** and the trifluoropropyl ester **8e** were shown to be stable in plasma for at least 1 h. The metabolite of compound **8d** was identified as compound **3** by LC−MS/MS, and the metabolite **3** showed no inhibitory activity against EGF shedding and MMPs as expected.

These results suggested that the trifluoroethoxy group and the difluoroethoxy group attached to the phosphorus atom may act as better electron-withdrawing groups. As a result, an increase in the positive charge on the phosphorus atom would facilitate enzymatic hydrolyses of the compounds. Such a dramatic change of the hydrolysis rate for a phosphate monoester by an alkaline phosphatase has been reported previouly.[24] The study suggested that the hydrolysis rates were linearly dependent on the pK_a values of the leaving groups, which are closely related to the positive charge on the phosphorus atom. To ascertain our assumption, the electronic environments at the phosphorus atoms of these compounds were evaluated by measuring ^{31}P NMR. As shown in Figure 1, the chemical shifts were moved downfield by the substitution of fluorine atoms. The chemical shift of compound **8d** was 25.9 ppm, which was 2.5 ppm lower field than that of compound **8a**. The

Table 1. In Vitro Potency and Stability of the Phosphonamide Derivatives[a]

compd	R	IC_{50} (μM) HB-EGF	IC_{50} (μM) AR	K_i (nM) MMP-1	K_i (nM) MMP-3	K_i (nM) MMP-9	stability in human plasma (% compd remaining after 60 min)
8a	Et	0.23	0.35	4.59	5.20	5.05	100
9a	Et	>10	NT	>850	>650	>800	NT
8b	CH_2CH_2F	0.18	0.47	10.5	9.07	10.5	96
8c	CH_2CHF_2	0.51	1.47	6.00	6.67	4.97	0 ($t_{1/2} \approx 10$ min)
8d	CH_2CF_3	0.73	0.95	6.57	6.75	3.68	0 ($t_{1/2} < 1$min)
8e	$CH_2CH_2CF_3$	0.31	0.97	2.21	3.33	4.46	99
3		>10	NT	>850	>650	>800	NT

[a] See Experimental Section for details of experimental assays. NT = not tested.

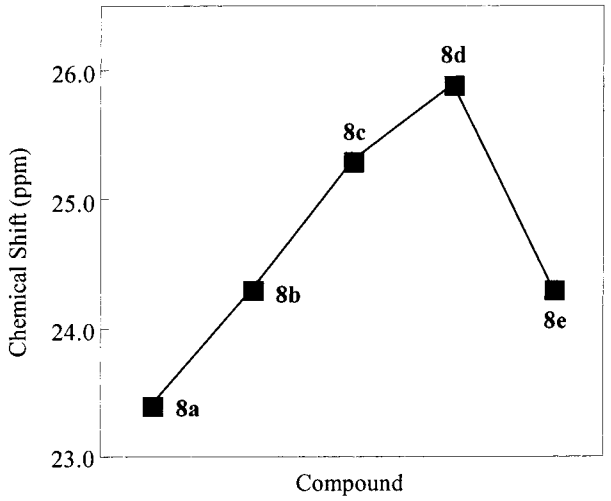


Figure 1. Chemical shifts of the ^{31}P NMR spectra of the phosphonamide derivatives.

resonance of compound **8c** also moved downfield by 1.9 ppm compared with that of compound **8a**. This lowering of the chemical shifts presumably resulted from an increase in the positive charge on the phosphorus atom. However, the extension of the methylene chain moved the chemical shift of compound **8e** to higher field similar to that of the monofluoroethyl ester **8b**. These results suggested that the degradation of the phosphonamide inhibitors in plasma would be dependent on the electronic environments on the phosphorus atom.

From the results of the studies for the enzyme inhibition and the plasma stability assay, compounds **8c** and **8d** were found to have great potential as the antedrug, and thus these compounds were subjected to further in vivo experiments.

To evaluate the antipsoriatic activities of compounds, we investigated the effects of these inhibitors on TPA-induced hyperplasia in murine skin, which is one of the psoriatic models.[25,26] It is well-known that topical application of phorbol esters induces cutaneous inflammation and epidermal hyperproliferation. As shown in Table 2, TPA induced a significant increase in epidermal thickness (62.2 μm), compared with acetone treatment (23.2 μm). Topical application of **8a** effectively suppressed TPA-induced inflammation with a 45.6% decrease at a dose of 100 μg/head. Compounds **8c** and **8d** also significantly inhibited TPA-induced hyperplasia (57.7% and 81.3% decrease, respectively). These results suggested that compounds **8c** and **8d** existed in an active form at a target site, although these compounds were promptly metabolized in plasma. It was noteworthy that **8c** and **8d** showed more potent antiinflammatory activities than the parent stable compound **8a**. The

Table 2. Effect of Phosphonamide Derivatives on TPA-Induced Epidermal Hyperplasia[a]

compd	epidermal thickness (μm)	% decrease
TPA + vehicle	62.2 ± 1.5	
TPA + **8a**	44.4 ± 7.3[b]	45.6
TPA + **8c**	39.7 ± 3.4[c]	57.7
TPA + **8d**	30.5 ± 4.8[c]	81.3
vehicle only	23.2 ± 1.7[c]	

[a] See Experimental Section for details of experimental assays. Data represent the mean ± standard error of five animals. Significant difference from (TPA + vehicle) treated group. [b] $p < 0.05$. [c] $p < 0.01$ (Student's *t*-test).

reason for this difference may be that the fluorine atoms in the phosphonamide ester moiety would improve the pharmacokinetic profiles, such as skin permeability, and this resulted in more potent antiinflammatory effects. In fact, the trifluorinated compound **8d** exhibited the most potent inhibitory activity for TPA-induced epidermal hyperplasia (30.5 μm, 81.3% decrease). Furthermore, compound **8d** inhibited TPA-induced hyperplasia in a dose-dependent manner.[27] These data indicated that compounds **8c** and **8d** would be stable in the epidermal and would regulate EGF production to suppress phorbol-induced inflammation and epidermal hyperplasia. Considering the potent topical antiinflammatory activity and its rapid decomposition property in plasma, it was strongly suggested that compound **8d** would have great potential as the antedrug, and thus, topical use of compound **8d** was the most promising candidate for the treatment of psoriasis.

Conclusions

In the present paper, we provided a novel strategy for the application of a metalloproteinase inhibitor as the antedrug. The phosphonamide-based metalloproteinase inhibitors showed potent activities against HB-EGF and AR shedding, which seem to be involved in hyperproliferation of epidermal keratinocytes. The trifluoroethyl ester derivative **8d** showed extremely rapid decomposition in human plasma. The difluoroethyl ester **8c** also showed moderate decomposition; however, the ethyl ester **8a** and monofluoroethyl ester **8b** were stable in plasma. The results of ^{31}P NMR suggested that an increase in the positive charge on the phosphorus atom would strongly affect the decomposition rate. Topical applications of compounds **8c** and **8d** significantly reduced TPA-induced inflammation and epidermal hyperplasia. These results suggested that compound **8d** may represent a novel approach to the treatment of psoriasis.

Experimental Section

General Methods and Materials. All commercially available starting materials and solvents were reagent grade. Melting points were uncorrected. ^{1}H NMR spectra were measured at 250 MHz on a Bruker DPX-250 spectrometer using $CDCl_3$ or DMSO-d_6 as the solvent. ^{31}P NMR spectra were recorded at 243 MHz on a Bruker DRX-600 spectrometer using DMSO as the solvent with 85% H_3PO_4 as an external reference. TPA was purchased from Sigma (St. Louis, MO). Mass spectra were determined on a Perceptive Biosystems Voyager-DETM RP spectrometer. Elemental analysis was conducted by Sumika Chemical Analysis Service, Ltd., Japan, and the results are within ±0.4% of the calculated values unless otherwise noted.

General Procedure for Preparing Phosphonamide Derivatives (Method A). (3*R*)-2-*tert*-Butoxycarbonyl-1,2,3,4-tetrahydroisoquinole-3-carboxylic Acid. To a solution of (3*R*)-1,2,3,4-tetrahydroisoquinole-3-carboxylic acid hydrochlorde[28] (17.1 g, 80 mmol) in 200 mL of 50% aqueous 1,4-dioxane was added sodium carbonate (17.0 g, 160 mmol) and $(Boc)_2O$ (21.0 g, 96 mmol), and the mixture was stirred at room temperature overnight. The reaction mixture was concentrated to a half-volume in vacuo, and the solution was acidified with 1 N HCl (pH 3). The solution was extracted with AcOEt, and the organic layer was washed successively with 1 N HCl and brine and dried over $MgSO_4$. The solvent was evaporated to give the title compound (13.5 g, 61%): ^{1}H NMR (DMSO-d_6) δ 1.37 (s, 4.5H), 1.44 (s, 4.5H), 3.00−3.25 (m, 2H), 4.35−4.60 (m, 2H), 4.60−4.70 (m, 0.5H), 4.80−4.90 (m, 0.5H), 7.15−7.25 (m, 4H).

(3*R*)-*N*-Benzyloxy-2-*tert*-butoxycarbonyl-1,2,3,4-tetrahydroisoquinole-3-carboxamide. To a solution of (3*R*)-2-*tert*-butoxycarbonyl-1,2,3,4-tetrahydroisoquinole-3-carboxylic acid (13.5 g, 48.7 mmol) in DMF (200 mL) was added WSC (10.4 g, 54 mmol), HOBt (8.3 g, 54 mmol), *O*-benzylhydroxylamine hydrochloride (8.6 g, 54 mmol), and triethylamine (5.5 g, 54 mmol), and the mixture was stirred at room temperature overnight. The reaction mixture was diluted with AcOEt (200 mL), washed successively with 1 N HCl, saturated $NaHCO_3$, and brine, and dried over $MgSO_4$. The solvent was evaporated, and the residue was recrystallized from AcOEt−hexane to give the title compound (7.6 g, 41%): ^{1}H NMR ($CDCl_3$) δ 1.41 (s, 4.5H), 1.52 (s, 4.5H), 3.15−3.35 (m, 2H), 4.45−4.75 (m, 2H), 4.80−4.85 (m, 0.5H), 5.00−5.20 (m, 2H), 5.15−5.25 (m, 0.5H), 7.05−7.40 (m, 9H).

(3*R*)-*N*-Benzyloxy-1,2,3,4-tetrahydroisoquinole-3-carboxamide (6). (3*R*)-*N*-benzyloxy-2-*tert*-butoxycarbonyl-1,2,3,4-tetrahydroisoquinole-3-carboxamide (7.6 g, 19.9 mmol) was dissolved in 4 N HCl/AcOEt (50 mL), and the solution was stirred at room temperature for 2 h. The precipitates were filtered and washed with Et_2O. The solids were dissolved in water, neutralized with $NaHCO_3$, and extracted with AcOEt. The organic layer was washed with brine and dried over $MgSO_4$. Removal of the solvent gave the title compound (4.6 g, %): ^{1}H NMR (DMSO-d_6) δ 2.70−2.80 (m, 2H), 3.30−3.35 (m, 1H), 3.75−3.95 (m, 2H), 4.80 (s, 2H), 6.95−7.15 (m, 4H), 7.30−7.45 (m, 5H).

Synthesis of (3*R*)-*N*-Hydroxy-2-[(*R* and *S*)-ethoxy(4-methoxyphenyl)phosphoryl]-1,2,3,4-tetrahydroisoquinoline-3-carboxamide (8a and 9a). Diastereomers of (3*R*)-*N*-Benzyloxy-2-[(*RS*)-ethoxy(4-methoxyphenyl)phosphoryl]-1,2,3,4-tetrahydroisoquinoline-3-carboxamide (7a). To a solution of 4-methoxyphenylphosphonic dichloride (398 mg, 1.77 mmol) in 5 mL of pyridine was added (3*R*)-*N*-benzyloxy-1,2,3,4-tetrahydroisoquinole-3-carboxamide **6**,[13] and the mixture was stirred at room temperature for 15 min under an argon atmosphere. Then, ethanol (0.1 mL, 1.77 mmol) was added to the solution, and the mixture was stirred at room temperature for 1 h. The reaction mixture was concentrated in vacuo, and the remaining pyridine was completely removed by azeotropic evaporation with toluene. The residual oil was diluted with AcOEt, and the solution was washed successively with saturated $NaHCO_3$ and brine and dried over $MgSO_4$. The solvent was evaporated, and the residue was purified by column chromatography on silica gel, eluting with AcOEt to give the title compound as a diasteromeric mixture (ca. 2:1, 466 mg, 55%): ^{1}H NMR ($CDCl_3$) δ 1.19 (t, J = 7.1 Hz, 1H), 1.31 (t, J = 7.1 Hz, 2H), 2.75−2.95 (m, 1H), 3.30−3.45 (m, 1H), 3.81 (s, 2H), 3.85 (s, 1H), 3.90−4.30 (m, 4H), 4.45−4.55 (m, 0.3 H), 4.75−4.85 (m, 2H), 6.85−6.95 (m, 3H), 7.05−7.20 (m, 3H), 7.25−7.35 (m, 5H), 7.45−7.70 (m, 2H), 9.71 (br s, 0.3H), 10.11 (br s, 0.7H).

(3*R*)-*N*-Hydroxy-2-[(*R* and *S*)-ethoxy(4-methoxyphenyl)phosphoryl]-1,2,3,4-tetrahydroisoquinoline-3-carboxamide (8a and 9a). A mixture of compound **7a** (466 mg, 0.97 mmol) and 10% Pd−C (40 wt %, prewashed with EtOH) in EtOH (20 mL) was stirred at room temperature under H_2 for 3 h. Pd−C was filtered off, and the filtrate was concentrated. The residue was purified by HPLC (YMC-ODS, CH_3CN/water 30:70) to afford two diastereomerically pure hydroxamic acids. **8a** (80 mg, 21%) was eluted first, and then **9a** (127 mg, 34%) was eluted.

(3*R*)-*N*-Hydroxy-2-[(*R*)-ethoxy(4-methoxyphenyl)phosphoryl]-1,2,3,4-tetrahydroisoquinoline-3-carboxamide (8a): colorless solids; ^{1}H NMR (DMSO-d_6) δ 1.14 (t, J = 7.0 Hz, 3H), 2.90−3.05 (m, 2H), 3.65−3.80 (m, 1H), 3.79 (s, 3H), 3.85−4.00 (m, 1H), 4.14 (dd, J = 7.6 and 16.1 Hz, 1H), 4.30 (dd, J = 4.5 and 16.1 Hz, 1H), 4.35−4.45 (m, 1H), 7.02 (dd, J = 3.1 and 8.9 Hz, 2H), 7.00−7.15 (m, 4H), 7.69 (dd, J = 8.8 and 12.4 Hz, 2H), 8.78 (br s, 1H), 10.57 (br s, 1H); ^{31}P NMR δ 23.4; MALDI-TOF MS 429 $[M + K]^+$, 413 $[M + Na]^+$, 391 $[M + H]^+$. Anal. ($C_{19}H_{23}N_2O_5P$) C, H, N.

(3*R*)-*N*-Hydroxy-2-[(*S*)-ethoxy(4-methoxyphenyl)phosphoryl]-1,2,3,4-tetrahydroisoquinoline-3-carboxamide (9a): recrystallization from EtOH−CH_3CN gave colorless crystals; mp 165−166 °C; ^{1}H NMR (DMSO-d_6) δ 1.31 (t, J = 7.0 Hz, 3H), 2.90−3.10 (m, 2H), 3.75 (s, 3H), 3.90−4.20 (m, 3H), 4.40−4.60 (m, 2H), 6.90−7.10 (m, 6H), 7.54 (dd, J = 8.8 and 12.3 Hz, 2H), 8.76 (br s, 1H), 10.54 (br s, 1H); MALDI-TOF MS 429 $[M + K]^+$, 413 $[M + Na]^+$, 391 $[M + H]^+$. Anal. ($C_{19}H_{23}N_2O_5P$) C, H, N.

Compounds **8b**, **9b**, **8e**, and **9e** were also synthesized using a procedure similar to the procedure for the preparation of **8a** and **9a**.

(3*R*)-*N*-Hydroxy-2-[(*R*)-(2-fluoroethoxy)(4-methoxyphenyl)phosphoryl]-1,2,3,4-tetrahydroisoquinole-3-carboxamide (8b): colorless solids; 36%; ^{1}H NMR (DMSO-d_6) δ 2.85−3.00 (m, 2H), 3.81 (s, 3H), 3.85−4.35 (m, 4H), 4.40−4.50 (m, 2H), 4.55−4.70 (m, 1H), 7.00−7.15 (m, 6H), 7.73 (dd, J = 8.8 and 12.6 Hz, 2H), 8.78 (br s, 1H), 10.59 (br s, 1H); ^{31}P NMR δ 24.3; MALDI-TOF MS 447 $[M + K]^+$, 431 $[M + Na]^+$, 409 $[M + H]^+$. Anal. ($C_{19}H_{22}FN_2O_5P$) C, H, N.

(3*R*)-*N*-Hydroxy-2-[(*S*)-(2-fluoroethoxy)(4-methoxyphenyl)phosphoryl]-1,2,3,4-tetrahydroisoquinole-3-carboxamide (9b): colorless solids; 12%; ^{1}H NMR (DMSO-d_6) δ 2.90−3.15 (m, 2H), 3.76 (s, 3H), 3.98 (dd, J = 7.5 and 17.5 Hz, 1H), 4.10−4.50 (m, 3H), 4.50−4.70 (m, 2H), 4.70−4.85 (m, 1H), 6.90−7.00 (m, 3H), 7.00−7.15 (m, 3H), 7.56 (dd, J = 8.5 and 12.3 Hz, 2H), 8.78 (s, 1H), 10.58 (s, 1H); MALDI-TOF MS 447 $[M + K]^+$, 431 $[M + Na]^+$, 409 $[M + H]^+$. Anal. ($C_{19}H_{22}FN_2O_5P$) C, H, N.

(3*R*)-*N*-Hydroxy-2-[(*R*)-(3,3,3-trifluoropropoxy)(4-methoxyphenyl)phosphoryl]-1,2,3,4-tetrahydroisoquinole-3-carboxamide (8e): colorless solids; 35%; ^{1}H NMR (DMSO-d_6) δ 2.50−2.70 (m, 2H), 2.85−3.05 (m, 2H), 3.80 (s, 3H), 3.80−3.95 (m, 1H), 4.05−4.20 (m, 2H), 4.25−4.35 (m, 1H), 4.35−4.45 (m, 1H), 7.00−7.15 (m, 6H), 7.70 (dd, J = 8.7 and 12.5 Hz, 2H), 8.80 (s, 1H), 10.60 (s, 1H); ^{31}P NMR δ 24.3; MALDI-TOF MS 497 $[M + K]^+$, 481 $[M + Na]^+$, 459 $[M + H]^+$. Anal. ($C_{20}H_{22}F_3N_2O_5P$) C, H, N.

(3*R*)-*N*-Hydroxy-2-[(*S*)-(3,3,3-trifluoropropoxy)(4-methoxyphenyl)phosphoryl]-1,2,3,4-tetrahydroisoquinole-3-carboxamide (9e): colorless solids; 25%; ^{1}H NMR (DMSO-d_6) δ 2.70−2.85 (m, 2H), 2.95−3.15 (m, 2H), 3.75 (s, 3H), 3.92 (dd, J = 7.5 and 16.1 Hz, 1H), 4.20−4.50 (m, 3H), 4.55−4.60 (m, 1H), 6.90−7.00 (m, 3H), 7.00−7.15 (m, 3H), 7.53 (dd, J = 8.8 and 12.5 Hz, 2H), 8.78 (s, 1H), 10.59 (br s, 1H); MALDI-

TOF MS 497 $[M + K]^+$, 481 $[M + Na]^+$, 459 $[M + H]^+$. Anal. ($C_{20}H_{22}F_3N_2O_5P$) C, H, N.

General Procedure for Preparing Phosphonamide Derivatives (Method B). Synthesis of (3*R*)-*N*-Hydroxy-2-[(*R* and *S*)-(2,2,2-trifluoroethoxy)(4-methoxyphenyl)-phosphoryl]-1,2,3,4-tetrahydroisoquinoline-3-carboxamide (8d and 9d). (4-Methoxyphenyl)phosphonic Acid Bis(2,2,2-trifluoroethyl) Ester. To a solution of 2,2,2-trifluoroethanol (466 mg, 4.66 mmol) in dry THF (50 mL) was added NaH (123 mg, 5.13 mmol), and the mixture was stirred at room temperature under an argon atmosphere for 30 min. Then, 4-methoxyphenylphosphonic dichloride (500 mg, 2.22 mmol) was added to the mixture, and the stirring was continued overnight. The reaction mixture was quenched with 1 N HCl (50 mL) and extracted with AcOEt. The organic layer was washed with saturated $NaHCO_3$ and brine and dried over $MgSO_4$. The solvent was evaporated to give the title compound as a colorless oil (650 mg, 83%): ^{1}H NMR ($CDCl_3$) δ 3.87 (s, 3H), 4.25−4.50 (m, 4H), 7.01 (dd, J = 3.9 and 8.9 Hz, 2H), 7.76 (dd, J = 8.9 and 13.6 Hz, 2H).

(4-Methoxyphenyl)phosphonic Acid Mono(2,2,2-trifluoroethyl) Ester. To a solution of (4-methoxyphenyl)-phosphonic acid bis(2,2,2-trifluoroethyl) ester (650 mg, 1.84 mmol) in 1,4-dioxane (7 mL) was added 1.8 N KOH solution (3 mL), and the mixture was refluxed for 2 h. The reaction mixture was acidified with 1 N HCl and extracted with AcOEt. The organic layer was washed with brine and dried over $MgSO_4$. The solvent was evaporated to give the title compound as a colorless oil (477 mg, 96%): ^{1}H NMR ($CDCl_3$) δ 3.86 (s, 3H), 4.15−4.30 (m, 2H), 6.97 (dd, J = 3.7 and 8.9 Hz, 2H), 7.73 (dd, J = 8.9 and 13.5 Hz, 2H).

Diastereomers of (3*R*)-*N*-Benzyloxy-2-[(*RS*)-(2,2,2-trifluoroethoxy)(4-methoxyphenyl)phosphoryl]-1,2,3,4-tetrahydroisoquinoline-3-carboxamide (7d). A mixture of (4-methoxyphenyl)phosphonic acid mono(2,2,2-trifluoroethyl) ester (477 mg, 1.77 mmol) and a catalytic amount of DMF (a few drops) in thionyl chloride (5 mL) was refluxed for 2 h. The reaction mixture was concentrated in vacuo, and the residual thionyl chloride was completely removed by azeotropic evaporation with toluene to afford 580 mg of crude monochloride, which was used for the next reaction without further purification. To a solution of the above product in 5 mL of dry THF was added (3*R*)-*N*-benzyloxy-1,2,3,4-tetrahydroisoquinole-3-carboxamide[13] (300 mg, 1.06 mmol) and diisopropylethylamine (363 μL, 2.1 mmol), and the mixture was stirred at room temperature under an argon atmosphere overnight. The reaction mixture was concentrated, and the residual oil was diluted with AcOEt. The solution was washed successively with water, saturated $NaHCO_3$, and brine and dried over $MgSO_4$. The solvent was evaporated, and the residue was purified by column chromatography on silica gel, eluting with AcOEt to give 255 mg of crude product as a diasteromeric mixture (1:1). This crude product was used for the next reaction without further purification.

(3*R*)-*N*-Hydroxy-2-[(*R* and *S*)-(2,2,2-trifluoroethoxy)(4-methoxyphenyl)phosphoryl]-1,2,3,4-tetrahydroisoquinoline-3-carboxamide (8d and 9d). A mixture of compound **7d** (255 mg) and 10% Pd−C (200 mg, prewashed with EtOH) in EtOH (10 mL) was stirred at room temperature under H_2 for 3 h. Pd−C was filtered off, and the filtrate was concentrated. The residue was purified by HPLC (YMC-Pack ODS, CH_3CN/water 40:60) to afford two diastereomerically pure hydroxamic acids. The (*R*)-isomer **8d** (25 mg, 12%) was eluted first, and then the (*S*)-isomer **9d** (28 mg, 13%) was eluted.

(3*R*)-*N*-Hydroxy-2-[(*R*)-(2,2,2-trifluoroethoxy)(4-methoxyphenyl)phosphoryl]-1,2,3,4-tetrahydroisoquinoline-3-carboxamide (8d): colorless solids; ^{1}H NMR (DMSO-d_6) δ 2.90−3.10 (m, 2H), 3.82 (s, 3H), 4.10−4.20 (m, 1H), 4.25−4.35 (m, 2H), 4.35−4.45 (m, 1H), 4.50−4.65 (m, 1H), 7.00−7.15 (m, 6H), 7.75 (dd, J = 8.8 and 12.8 Hz, 2H), 8.80 (br s, 1H), 10.63 (br s, 1H); ^{31}P NMR δ 25.9; MALDI-TOF MS 483 $[M + K]^+$, 467 $[M + Na]^+$, 445 $[M + H]^+$. Anal. ($C_{19}H_{20}F_3N_2O_5P$) C, H, N.

(3*R*)-*N*-Hydroxy-2-[(*S*)-(2,2,2-trifluoroethoxy)(4-methoxyphenyl)phosphoryl]-1,2,3,4-tetrahydroisoquinoline-3-carboxamide (9d): colorless solids; ^{1}H NMR (DMSO-d_6) δ 3.00−3.20 (m, 2H), 3.77 (s, 3H), 3.85−3.95 (m, 1H), 4.30−4.40 (m, 1H), 4.55−4.90 (m, 3H), 6.95−7.05 (m, 3H), 7.05−7.15 (m, 3H), 7.56 (dd, J = 8.8 and 12.6 Hz, 2H), 8.83 (br s, 1H), 10.66 (br s, 1H); MALDI-TOF MS 483 $[M + K]^+$, 467 $[M + Na]^+$, 445 $[M + H]^+$. Anal. ($C_{19}H_{20}F_3N_2O_5P$) C, H, N.

Compounds **8c** and **9c** were also synthesized using a procedure similar to the procedure for the preparation of **8d** and **9d**.

(3*R*)-*N*-Hydroxy-2-[(*R*)-(2,2-difluoroethoxy)(4-methoxyphenyl)phosphoryl]-1,2,3,4-tetrahydroisoquinole-3-carboxamide (8c): colorless solids; 30%; ^{1}H NMR (DMSO-d_6) δ 2.99−3.16 (m, 2H), 3.90 (s, 3H), 3.95−4.15 (m, 1H), 4.20−4.45 (m, 3H), 4.45−4.55 (m, 1H), 6.27 (tt, J = 3.2 and 54.3 Hz, 1H), 7.10−7.25 (m, 6H), 7.82 (dd, J = 8.7 and 12.6 Hz, 2H), 8.89 (s, 1H), 10.70 (s, 1H); ^{31}P NMR δ 25.3; MALDI-TOF MS 465 $[M + K]^+$, 449 $[M + Na]^+$, 427 $[M + H]^+$. Anal. ($C_{19}H_{21}F_2N_2O_5P$) C, H, N.

(3*R*)-*N*-Hydroxy-2-[(*S*)-(2,2-difluoroethoxy)(4-methoxyphenyl)phosphoryl]-1,2,3,4-tetrahydroisoquinole-3-carboxamide (9c): colorless solids; 38%; ^{1}H NMR (DMSO-d_6) δ 3.05−3.25 (m, 2H), 3.85 (s, 3H), 4.02 (dd, J = 7.1 and 16.3 Hz, 1H), 4.30−4.55 (m, 3H), 4.60−4.70 (m, 1H), 6.45 (tt, J = 3.4 and 54.6 Hz, 1H), 7.00−7.10 (m, 3H), 7.15−7.20 (m, 3H), 7.65 (dd, J = 8.8 and 12.5 Hz, 2H), 8.90 (s, 1H), 10.72 (s, 1H); MALDI-TOF MS 465 $[M + K]^+$, 449 $[M + Na]^+$, 427 $[M + H]^+$. Anal. ($C_{19}H_{21}F_2N_2O_5P$) C, H, N.

Shedding Assay for EGF Receptor Ligand−AP Fusion Protein. Expression plasmid of HB-EGF and human placental alkaline phosphatase (AP) fusion protein that were constructed as described previously[29] were a generous gift from Dr. Higashiyama (School of Medicine, Osaka University, Osaka, Japan). The plasmid was transfected into HT1080 cells (American Type Culture Collection, Rockville, MD) by lipofection using a lipofectamine system (Gibco/BRL, Gaithersburg, MD) according to the manufacturer's directions. Stable transfectants were selected by growth in G418. Stable transfectants of AR were established in the same way as those of HB-EGF.

Stable transfectants expressing EGF receptor ligand−AP fusion protein in MEM (containing 10% FCS) as a culture medium were seeded in 96-well plates at a density of 2×10^5 cells/well and incubated for 24 h. The cells were washed with PBS and preincubated with test compounds in MEM (containing 1% DMSO) for 30 min. TPA (60 nM) was added to stimulate inducible processing, and the plate was incubated for 60 min. A 0.1 mL aliquot of the supernatant was transferred to 96-well plates and heated for 10 min at 65 °C in order to inactivate endogenous alkaline phosphatases. A 0.1 mL of substrate solution (1 M diethanolamine, 0.01% $MgCl_2$, 1 mg/mL *p*-nitrophenyl phosphate, pH 9.8) was added to each well, and the plates were incubated for 1−2 h. AP activity was then determined by the measurement of absorbance at 405 nm with a microplate reader. The IC_{50} value was determined with different inhibitor concentrations by using GraphPad Prism, version 3.0 (GraphPad Software, Inc.).

Expression and Purification of Human Recombinant MMPs. DNA fragments coding the catalytic domain of human MMP-1 and human MMP-9 and a DNA fragment coding from the prodomain to the catalytic domain of human MMP-3 were amplified by polymerase chain reaction (PCR) from cDNA of HT1080 cells stimulated with 0.01 μM of TPA. A sequence for the appropriate restriction enzyme site was added to the 5′-end of each PCR primer. Amplified DNA fragments were cloned into a cloning vector and then introduced into a commercially available expression vector containing a His-6 tag sequence at the end of the N-terminus. Recombinant proteins were expressed in *E. coli* cells and purified by Ni-NTA resin (QIAGEN INC.) and refolded. Recombinant MMP-3 was activated by incubating with 1 mM *p*-aminophenylmercuric acetate for 1 h at 37 °C.

MMP Inhibition Assay. Recombinant MMP-1, −3, and −9 were used at final concentrations of 5, 6.5, and 24 nM, respectively. The assays were performed using the fluorogenic

substrate MOCAc-Pro-Leu-Gly-Leu-A2pr(Dnp)-Ala-Arg-NH_2 (Peptide Institute, Inc.) at a final concentration of 5 μM (MOCAc-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys(Dnp)-NH_2 [Peptide Institute, Inc.] was used for MMP-3). Typical assays were performed as follows. In a well of a 96-well half-area black microplate (COSTAR), an enzyme solution (25 μL) was incubated with 25 μL of a test compound solution (10 mM DMSO stock solution was diluted to the appropriate concentration with the assay buffer) in 50 mM Tris, 150 mM NaCl, 10 mM $CaCl_2$, 0.05% Brij-35, pH 7.5, for 10 min at 37 °C. Then the reaction was initiated by adding 50 μL of the substrate solution to the 96-well plate, and incubation was continued for 2 h at 37 °C (3 h for MMP-9). The increase in fluorescence due to cleavage of the substrate was measured with a fluorescence microplate reader (excitation/emission = 320/405 nm, Polarstar; BMG LabTechnologies, Germany). K_i values were calculated by nonlinear regression analysis using the percent inhibition and K_m values of the substrates for each MMP with the program GraphPad Prism (GraphPad Software, Inc., San Diego, CA).

Stability Assay in Human Plasma. A stock solution of test compound (final concentration of 10 μM) was added to plasma (500 μL) obtained from human (healthy volunteers) or mice (male ddY, 6 weeks old), and the assay mixture was incubated for 1 h at 37 °C. A 100 μL aliquot of the assay mixture was then mixed with 500 μL of ice-cold acetonitrile and centrifuged at 3000 rpm for 10 min. The solvent of the supernatant was replaced with an eluent, and the stability of the compound was then determined by HPLC on a column of L-column (4.6 mm × 150 mm, Chemicals Evaluation Research Institute, Japan) with water−acetonitrile eluent. The remaining percent was calculated from the average of two measurements.

Inhibition Assay for TPA-Induced Epidermal Hyperplasia Model. Backs of mice were shaved by an electric clipper and treated with depilatory cream (Eva cream, Tokyo Tanabe, Tokyo). Three days later, the mice that displayed no evidence of hair regrowth were used for experiments. A total of 20 μL of TPA (1 nM) or vehicle (acetone) was applied to the skin surface of the backs in an area of ca. 1 cm^2 using a micropipet. A minute after TPA application or 1 h prior to TPA application, a test compound or vehicle (acetone) was topically administrated to the same area where it was TPA-treated (day 0). The treatment of the test compound or vehicle was repeated on days 1 and 2. On day 3, the mice were sacrificed, and skin tissues treated with TPA−test compound were removed. The tissue specimen was made and stained by hematoxylin-eosin. The epidermal thickness was measured as the distance from the bottom of the stratum corneum to the bottom of the basal layer using an ocular microscope with a magnification of 400×. The measurement was performed at 10 different fields within a width of 6 mm, and the mean was calculated.

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