

First Synthesis and Determination of the Absolute Configuration of Sulphostin, a Novel Inhibitor of Dipeptidyl Peptidase IV

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Sulphostin, a novel dipeptidyl peptidase IV (DPP-IV) inhibitor, was isolated from the culture broth of *Streptomyces* sp. MK251-43F3. Determination of the absolute configurations of two asymmetric atoms using the natural product was not achieved due to the small amount of the compound obtained. We synthesized four possible stereoisomers of sulphostin from D- or L-ornithine and compared their physicochemical and biological data to naturally isolated sulphostin. As a result, the absolute configurations at C-3 and the phosphorus atom of sulphostin were determined to be *S* and *R*, respectively, by X-ray crystallography. Synthetic sulphostin and its C-3 epimer have strong inhibitory activities against DPP-IV, IC₅₀ values of which are 6.0 and 8.9 ng/mL, respectively. Thus it appears that the configuration of the phosphorus atom is primarily responsible for the activity; in contrast, the configuration of C-3 does not appear to affect the activity.

Dipeptidyl peptidase IV (DPP-IV, CD26, EC 3.4.14.5) is a cell-surface serine exopeptidase that selectively cleaves dipeptides from the N-terminus of polypeptide, provided that proline or alanine is the penultimate residue.¹ This enzyme cleaves several important polypeptides such as RANTES (regulated on activation, normal T cells expressed and secreted),² substance P,³ and glucagon-like peptide-1 (7-36 amide, GLP-1)⁴ in vitro. GLP-1 is one of the most noteworthy insulin-releasing hormones and is inactivated by DPP-IV. Therefore, some DPP-IV inhibitors were examined extensively in vivo,^{5,6} and clinical trials were carried out to examine their use as a type 2 diabetic agent.^{7,8} In the immune system, CD26, which is identical to DPP-IV, is expressed primarily on the surface of T cells and was shown to be a thymocyte-costimulating antigen in several studies.^{9,10} DPP-IV inhibitors are therefore also expected to be therapeutic agents for immune-related disorders.^{11,12}

We recently isolated a strong DPP-IV inhibitor called sulphostin from the culture broth of *Streptomyces* sp. MK251-43F3 (Figure 1).¹³ Isolated sulphostin showed a clear primary signal at 6.0 ppm and a minor signal at 6.3 ppm in the ³¹P NMR spectrum, indicating the existence of a stereoisomer. We assumed that the 6.0 ppm resonance in the ³¹P NMR spectrum was the signal of sulphostin and the 6.3 ppm resonance in the ³¹P NMR spectrum was the signal of the stereoisomer that occurred by epimerization of sulphostin during the isolation process.¹³ Because the amount of the product isolated from the culture broth was small and it was a mixture of stereoisomers, a detailed investigation of the configuration of sulphostin has not been performed. We report herein on the synthesis of four possible stereoisomers of sulphostin and the determination of the absolute configuration of the natural product by a comparison of NMR data and the DPP-IV inhibitory activities of the four synthetic compounds, in which the configurations were determined by X-ray crystallography.

Results

Synthesis. Sulphostin and its stereoisomers consist of an ornithine lactam and a characteristic amino(sulfo-

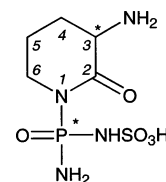
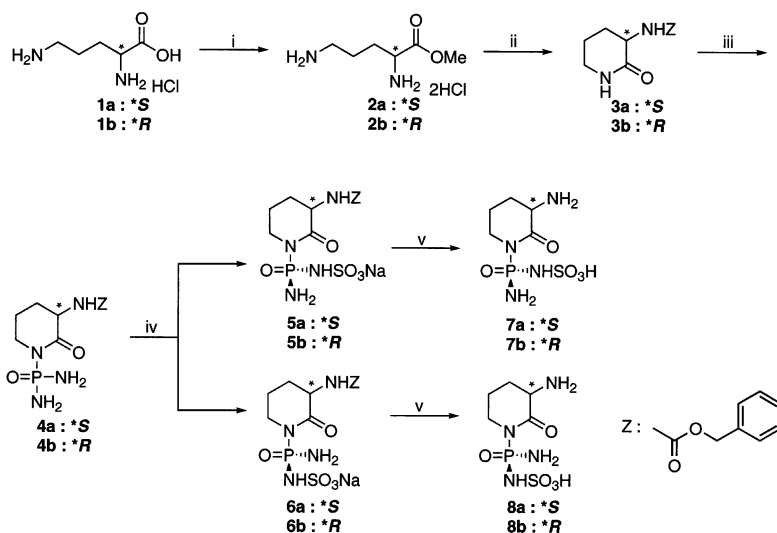


Figure 1. Structure of sulphostin.

amino)phosphinyl group. The synthesis of the amino(sulfoamino)phosphinyl group has not been reported yet. We considered that this functional group could be introduced by two-step reactions: diaminophosphinylation with phosphoryl chloride followed by treatment with ammonia and then sulfonation with sulfur trioxide. The separation of the diastereomers generated from the asymmetric phosphorus atom was expected to be possible by the usual chromatography techniques.

The synthetic method used in obtaining the four possible stereoisomers of sulphostin is shown in Scheme 1. For the synthesis of sulphostin and/or its stereoisomers **7a** and **8a**, in which the C-3 configuration is *S*, L-ornithine hydrochloride was used as the starting material. L-Ornithine hydrochloride was esterified by thionyl chloride in methanol to give L-ornithine methyl ester dihydrochloride (**2a**) and then cyclized into the lactam by neutralization with sodium hydrogen carbonate. The amino group was then protected as a urethane with benzyl chloroformate. Subsequent reaction of the lactam amide of (3*S*)-3-benzyloxycarbonyl-amino-2-piperidinone (**3a**) with *n*-butyllithium, then with phosphoryl chloride, followed by liquid ammonia, resulted in **4a**. Treatment of compound **4a** with pyridine/sulfur trioxide complex,¹⁴ followed by sodium hydrogen carbonate, gave the diastereomers **5a** and **6a**. The separation of this mixture of diastereomers was performed by Diaion HP-20SS column chromatography. However, this separation procedure proved to be much more difficult than originally anticipated, and the procedure had to be repeated in order to obtain optically pure material. Diaion HP-20SS column chromatography was carried out using water–30% MeOH/water as eluent, detecting by reversed-phase HPLC analysis, and the desired compounds were collected in the fractions of 20–25% MeOH/water. Almost all the fractions were mixtures of **5a** and **6a** in different ratios. Therefore,

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Scheme 1. Synthesis of Sulphostin and Its Three Stereoisomers

the fractions were roughly divided into 8 fraction groups based on the ratio of diastereomers **5a** and **6a**, and then each fraction group was resubjected to chromatography. The procedure was repeated to give each diastereomer **5a** and **6a** of >90% de. Owing to the poor separation of sodium salts using column chromatography, we used organic chiral amine salts, expecting good separation and facile desalination in the final process. (*S*)-(-)-1-Phenylethylamine was selected as an organic amine for purification of each diastereomer. Each enriched diastereomer **5a** and **6a** was treated with (*S*)-(-)-1-phenylethylamine hydrochloride to give the (*S*)-(-)-1-phenylethylamine salt of **5a** and **6a**, which was applied to Diaion HP-20SS column chromatography. The column was eluted with water–70% MeOH/water, and the ammonium salt was collected with 45–60% MeOH/water. The chromatography was repeated once more, and sodium chloride and excess (*S*)-(-)-1-phenylethylamine hydrochloride could be removed; however, the optical purity was not significantly improved. Therefore, a further chromatographic separation was required, and column chromatography using Sephadex LH-20 eluted with MeOH was carried out. Deprotection of the optically pure (*S*)-(-)-1-phenylethylamine salt was carried out with catalytic palladium-black under hydrogen atmosphere. The target compounds **7a** and **8a** were obtained after the removal of (*S*)-(-)-1-phenylethylamine with Diaion HP-20SS column chromatography and recrystallization. Single-crystal X-ray structure analyses of the synthetic compounds **7a** and **8a** gave their absolute configurations as 3*S*, *R*_P and 3*S*, *S*_P, respectively (Figure 2).

Similarly, the compounds **7b** and **8b**, for which the C-3 configuration is *R*, were synthesized from D-ornithine hydrochloride (**1b**) as a starting material and by using (*R*)-(+)-1-phenylethylamine as an organic amine for the purification.

In Vitro Assay for DPP-IV. DPP-IV was prepared from rat kidney homogenate by ammonium sulfate precipitation (30–80%).¹⁵ The inhibitory activity of the synthetic compound was determined by minor modification of the method of Oya et al.¹⁵ Gly-Pro-β-naphthylamide was used as a substrate, and the absorbance at 525 nm of the chromophore complex of β-naphthylamine, which was hydrolyzed from the substrate, was measured.

Table 1. ³¹P NMR and DPP-IV IC₅₀ Values of Synthetic Compounds

compound	configuration (C-3, P)	³¹ P NMR (ppm)	IC ₅₀ (ng/mL)
7a	<i>S</i> , <i>R</i>	6.03	6.0
8a	<i>S</i> , <i>S</i>	6.31	100 000
7b	<i>R</i> , <i>R</i>	6.29	8.9
8b	<i>R</i> , <i>S</i>	6.01	24 000

Discussion

¹H, ¹³C, and ³¹P NMR and DPP-IV inhibitory activity were measured for the four synthetic compounds, and the

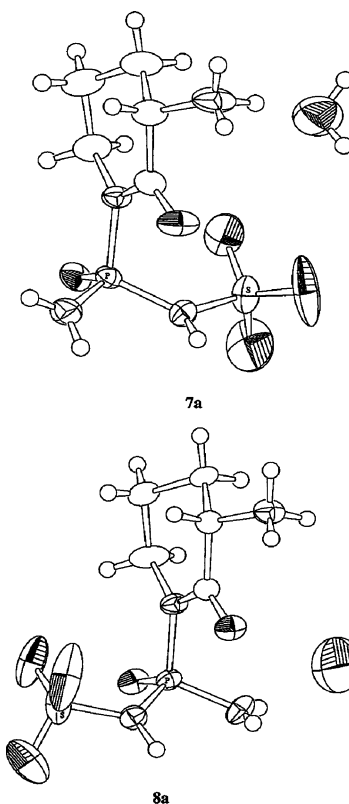


Figure 2. X-ray structures of compounds **7a** and **8a**.

data were compared with those of the natural product (Table 1). The synthetic compounds **7a** and **8b** showed a signal at 6.0 ppm in the ^{31}P NMR spectra, and these signals were compatible with the primary signal of natural sulphostin. Similarly, ^1H and ^{13}C NMR spectra were comparable. In contrast, ^{31}P NMR spectra of **7b** and **8a** showed a signal at 6.3 ppm, and these signals were compatible with the minor signal. As for the DPP-IV inhibitory activity, **7a** and **7b** showed significant activity with IC_{50} values of 6.0 and 8.9 ng/mL, respectively. In contrast, **8a** and **8b** exhibited only marginal activity. From these results, the natural product called sulphostin, which showed a primary signal at 6.0 ppm in the ^{31}P NMR spectrum, was found to be identical with the synthetic compound **7a** in a physicochemical and biological comparison. Therefore, the absolute configuration of sulphostin was determined to be C-3 *S* and phosphorus *R*, respectively.

From the structure–activity relationship between the stereochemistry of the synthetic compound and DPP-IV inhibition, it seems that the C-3 configuration does not appreciably affect the enzyme inhibitory activity. In contrast, the stereochemistry of phosphorus is definitive: only the compounds with *R* configuration exhibit inhibitory activity, and the compounds with *S* configuration seem to have no activity. Known synthetic DPP-IV inhibitors such as Xaa-2-cyanopyrrolidine,¹⁶ Xaa-boroPro,¹⁷ and Pro-pyrrolidine-2-PO(OAr)₂¹⁸ have a common Xaa-2-substituted pyrrolidine structure. They have important asymmetric atoms on the pyrrolidine ring. It is remarkable that sulphostin has a crucial asymmetric atom outside of the piperidinone ring. Because sulphostin and its C-3 epimer have strong inhibitory activity, they are expected to be excellent drug candidates.

Experimental Section

General Experimental Procedures. The melting points were determined with a Yanagimoto micro melting point apparatus. Optical rotations were measured with a Perkin-Elmer Model 241 polarimeter. IR spectra were recorded on a Horiba FT-200 FT infrared spectrometer. ^1H , ^{13}C , and ^{31}P NMR spectra were recorded with a JEOL GX-400 spectrometer or a JEOL JNM-A500 spectrometer. HRESIMS spectra were measured with a JEOL JMS-T100LC.

(3*S*)-3-Benzoyloxycarbonylamino-2-piperidinone (3a). To methanol (1.2 L) was added thionyl chloride (69.2 mL, 0.949 mol) at 0 °C, and the solution was stirred for 20 min. To the solution was added L-ornithine hydrochloride (80.00 g, 0.474 mol) by dividing into 4 portions, and the mixture was stirred for 3 h at 0 °C. The mixture was then allowed to warm to room temperature and was stirred for 19 h. After the reaction mixture was evaporated under reduced pressure, the residue was crystallized by trituration in Et₂O–*n*-hexane, and the collected crystals were washed with Et₂O to give L-ornithine methyl ester dihydrochloride. To a solution of the resultant ester salt in water (1.0 L) was added NaHCO₃ (119.56 g, 1.423 mol) at 0 °C by dividing into several portions, and the mixture was stirred for 15 h at room temperature. NaHCO₃ (59.78 g, 0.712 mol), THF (0.50 L), and benzyl chloroformate (81.3 mL, 0.569 mol) were added to this solution at 0 °C, and the mixture was stirred for 18 h at room temperature. The reaction mixture was separated, and the aqueous layer was extracted with CHCl₃. The combined organic layer was washed with saturated NaCl(aq), dried over anhydrous Na₂SO₄, and evaporated under reduced pressure. The residue was crystallized from Et₂O–*n*-hexane and was washed with Et₂O–*n*-hexane to give **3a** (105.60 g) in 90% yield from L-ornithine hydrochloride: mp 101–102 °C; $[\alpha]_D^{23}$ –18.5° (*c* 1.0, MeOH); IR (KBr) ν_{max} 3357, 3203, 1716, 1681 cm⁻¹; ^1H NMR (CDCl₃, 400 MHz) δ 7.28–7.38 (5H, m, Ph), 6.49 (1H, br s, H-1), 5.83 (1H, br d, *J* = 3.9 Hz, CHNHCOO), 5.11 (2H, s, OCH₂Ph), 4.09 (1H, td, *J* = 5.9,

11.7 Hz, H-3), 3.23–3.33 (2H, m, H-6), 2.48 (1H, m, H-4a), 1.80–1.96 (2H, m, H-5), 1.61 (1H, tdd, *J* = 11.2, 12.2, 5.4 Hz, H-4b); ^{13}C NMR (CDCl₃, 100 MHz) δ 171.5 (C-2), 156.4 (NHCOO), 136.4 (Ph), 128.5 (Ph \times 2), 128.1 (Ph \times 3), 66.8 (OCH₂-Ph), 51.7 (C-3), 41.7 (C-6), 27.7 (C-4), 21.0 (C-5); HRESIMS *m/z* 271.1053 [M + Na]⁺ (calcd for C₁₃H₁₆N₂NaO₃, 271.1059); anal. C 62.80%, H 6.51%, N 11.23%, calcd for C₁₃H₁₆N₂O₃, C 62.89%, H 6.50%, N 11.28%.

(3*R*)-3-Benzoyloxycarbonylamino-2-piperidinone (3b). The synthetic method was similar to that noted for compound **3a**. D-Ornithine hydrochloride (4.00 g, 23.7 mmol) was treated with thionyl chloride (3.45 mL, 47.5 mmol) in methanol (40 mL) to give D-ornithine methyl ester dihydrochloride. The resultant ester salt was treated with NaHCO₃ (5.98 g, 71.2 mmol) for cyclization at first, then with NaHCO₃ (2.99 g, 35.6 mmol) and benzyl chloroformate (4.06 mL, 28.4 mmol) for protection to give **3b** (4.60 g) in 78% yield from D-ornithine hydrochloride: mp 100.5–102 °C; $[\alpha]_D^{23}$ +18.5° (*c* 1.0, MeOH); IR (KBr) ν_{max} 3356, 3205, 1716, 1685 cm⁻¹; ^1H NMR (CDCl₃, 400 MHz) δ 7.27–7.37 (5H, m, Ph), 6.37 (1H, br s, H-1), 5.79 (1H, br s, CHNHCOO), 5.11 (2H, s, OCH₂Ph), 4.09 (1H, td, *J* = 5.9, 11.7 Hz, H-3), 3.26–3.34 (2H, m, H-6), 2.49 (1H, m, H-4a), 1.83–1.97 (2H, m, H-5), 1.61 (1H, tdd, *J* = 11.2, 12.7, 5.4 Hz, H-4b); ^{13}C NMR (CDCl₃, 100 MHz) δ 171.4 (C-2), 156.4 (NHCOO), 136.4 (Ph), 128.5 (Ph \times 2), 128.1 (Ph \times 3), 66.8 (OCH₂-Ph), 51.7 (C-3), 41.7 (C-6), 27.7 (C-4), 21.0 (C-5); HRESIMS *m/z* 271.1054 [M + Na]⁺ (calcd for C₁₃H₁₆N₂NaO₃, 271.1059).

(3*S*)-3-Benzoyloxycarbonylamino-1-diaminophosphinyl-2-piperidinone (4a). To a solution of compound **3a** (40.00 g, 0.161 mol) in anhydrous THF (450 mL) was slowly added 1.54 M *n*-butyllithium solution in *n*-hexane (99.0 mL, 0.152 mol) at –78 °C under nitrogen atmosphere. The mixture was stirred for 45 min at –78 °C. To the mixture was added a solution of phosphoryl chloride (15.0 mL, 0.161 mol) in anhydrous THF (20 mL), and the mixture was stirred for 45 min at –78 °C. After addition of liquid NH₃ (15.0 mL, 0.679 mol), the mixture was stirred for 5 min at –78 °C. The mixture was then allowed to warm to room temperature. To the reaction mixture was added saturated NaCl(aq) (500 mL). After water was added to the solution until the aqueous layer turned clear, the organic layer was separated. The aqueous layer was extracted 3 times with a mixture of CHCl₃ and MeOH (19:1). The combined organic layer was washed with saturated NaCl(aq), dried over anhydrous Na₂SO₄, and evaporated under reduced pressure. The solid residue was washed with a mixture of CHCl₃ and Et₂O (1:1) to give **4a** (19.63 g) in 37% yield: mp 155–158 °C; $[\alpha]_D^{23}$ –15.4° (*c* 1.0, MeOH); IR (KBr) ν_{max} 3344, 3296, 3253, 1699, 1645, 1211 cm⁻¹; ^1H NMR (DMSO-*d*₆, 400 MHz) δ 7.41 (1H, d, *J* = 8.8 Hz, CHNHCOO), 7.29–7.41 (5H, m, Ph), 5.03 (2H, s, OCH₂Ph), 4.21 (2H, br s, PNH₂), 4.16 (2H, br s, PNH₂), 4.10 (1H, td, *J* = 7.8, 11.2 Hz, H-3), 3.59 (1H, m, H-6a), 3.47 (1H, m, H-6b), 2.02 (1H, m, H-4a), 1.72–1.82 (2H, m, H-5), 1.63 (1H, m, H-4b); ^{13}C NMR (DMSO-*d*₆, 100 MHz) δ 172.9 (C-2), 156.0 (NHCOO), 137.0 (Ph), 128.2 (Ph \times 2), 127.7 (Ph \times 3), 65.3 (OCH₂Ph), 51.2 (C-3), 42.6 (C-6), 25.9 (C-4), 21.2 (C-5); HRESIMS *m/z* 349.1032 [M + Na]⁺ (calcd for C₁₃H₁₉N₄NaO₄P, 349.1042); anal. C 47.26%, H 5.87%, N 16.66%, calcd for C₁₃H₁₉N₄O₄P·1/4H₂O, C 47.20%, H 5.94%, N 16.94%.

(3*R*)-3-Benzoyloxycarbonylamino-1-diaminophosphinyl-2-piperidinone (4b). The synthetic method was similar to that noted for compound **4a**. Compound **3b** (4.00 g, 16.1 mmol) was treated with 1.55 M *n*-butyllithium solution in *n*-hexane (9.35 mL, 14.5 mmol) and phosphoryl chloride (2.72 g, 17.7 mmol) in anhydrous THF (10.0 mL), followed by liquid NH₃ (2.10 mL, 95.0 mmol) to give **4b** (1.50 g) in 29% yield: mp 155–158 °C; $[\alpha]_D^{23}$ +15.4° (*c* 1.0, MeOH); IR (KBr) ν_{max} 3342, 3296, 3251, 1699, 1645, 1211 cm⁻¹; ^1H NMR (DMSO-*d*₆, 400 MHz) δ 7.42 (1H, d, *J* = 8.9 Hz, CHNHCOO), 7.30–7.39 (5H, m, Ph), 5.03 (2H, s, OCH₂Ph), 4.21 (2H, br s, PNH₂), 4.16 (2H, br s, PNH₂), 4.10 (1H, td, *J* = 7.9, 11.9 Hz, H-3), 3.59 (1H, m, H-6a), 3.47 (1H, m, H-6b), 2.02 (1H, m, H-4a), 1.74–1.80 (2H, m, H-5), 1.63 (1H, m, H-4b); ^{13}C NMR (DMSO-*d*₆, 100 MHz) δ 173.0 (C-2), 156.1 (NHCOO), 137.0 (Ph), 128.3 (Ph \times 2), 127.79 (Ph \times 3), 127.76 (Ph), 65.4 (OCH₂Ph), 51.3 (C-3), 42.7 (C-6), 26.0

(C-4), 21.2 (C-5); HRESIMS m/z 349.1035 $[M + Na]^+$ (calcd for $C_{13}H_{19}N_4NaO_4P$, 349.1042).

(3*S*,*R*_P)-1-Amino(sulfoamino)phosphinyl-3-benzyloxy-carbonylamino-2-piperidinone Sodium Salt (5a) and (3*S*,*S*_P)-1-Amino(sulfoamino)phosphinyl-3-benzyloxy-carbonylamino-2-piperidinone Sodium Salt (6a). To a solution of compound **4a** (5.00 g, 15.3 mmol) in DMF (40 mL), dried with molecular sieves (4 Å) was added SO_3 -pyridine complex (3.17 g, 19.9 mmol) at 0 °C, and the mixture was stirred for 2 h at 6–8 °C. To the mixture were added water (50 mL) and $NaHCO_3$ (3.22 g, 38.3 mmol), and the mixture was stirred for 5 min at room temperature. The reaction mixture was diluted with water (200 mL) and was applied to a Diaion HP-20SS (500 mL) column. The column was washed with water (1000 mL) and then was eluted with water–30% MeOH/water at room temperature. The eluate was monitored by UV (254 nm) and was collected so that the weight of each fraction was 15 g. Each UV absorbing fraction was analyzed by reversed-phase HPLC. The desired compounds were collected in the fractions of 20–25% MeOH/water, and compound **6a** eluted slightly earlier than **5a**. The absorbing fractions were roughly divided into eight fraction groups based on the ratio of the diastereomer; the ratio (**6a**:**5a**) of each fraction group was about >20:1, 10:1, 4:1, 2:1, 1:2, 1:4, 1:10, and 1:>20, respectively. Each fraction group except the groups of >20:1 and 1:>20 was applied to Diaion HP-20SS column chromatography again, and each fraction was divided as mentioned above. Then, the fraction groups of analogous diastereomer ratio were combined and applied to column chromatography again. This procedure was repeated until sufficient amounts of concentrated diastereomer fractions were obtained. Evaporation of the fraction groups of >20:1 and 1:>20 under reduced pressure gave diastereomers **5a** and **6a** of >90% de, respectively: **5a** (0.80 g, 12% yield) and **6a** (1.04 g, 16% yield). To prepare analytical specimens of **5a** and **6a**, each optically pure (*S*)-(–)-1-phenylethylamine salt, which was purified in the next step, was changed into the sodium salt with sodium hydrogen carbonate and then purified by Diaion HP-20SS column chromatography (water–30% MeOH/water). The physicochemical data were obtained by using each resultant specimen.

Physicochemical data of 5a: $[\alpha]^{23}_D -65.5^\circ$ (c 1.0, H_2O); IR (KBr) ν_{max} 3386, 3303, 1726, 1647, 1219, 1059 cm^{-1} ; 1H NMR (D_2O , 400 MHz) δ 7.39–7.48 (5H, m, Ph), 5.12–5.21 (2H, m, OCH_2Ph), 4.32 (1H, dd, $J = 11.2, 6.8$ Hz, H-3), 3.75 (1H, m, H-6a), 3.61 (1H, m, H-6b), 2.20 (1H, m, H-4a), 2.02 (1H, m, H-5a), 1.90 (1H, m, H-5b), 1.76 (1H, tdd, $J = 11.7, 9.3, 6.8$ Hz, H-4b); ^{13}C NMR (D_2O , 100 MHz) δ 178.6 (C-2), 161.1 (NHCOO), 139.2 (Ph), 131.7 (Ph \times 2), 131.3 (Ph), 130.6 (Ph \times 2), 70.0 (OCH_2Ph), 54.8 (C-3), 47.4 (C-6), 28.5 (C-4), 23.3 (C-5); HRESIMS m/z 451.0427 $[M + Na]^+$ (calcd for $C_{13}H_{18}N_4Na_2O_7PS$, 451.0429); reversed-phase HPLC analysis, retention time 14.0 min (conditions: column, Senshu Pak PEGASIL ODS (4.6 \times 250 mm); column temperature, 40 °C; eluent, 0.1% trifluoroacetic acid(aq)/MeOH, 70:30; flow rate, 1.0 mL/min; detection, UV 205 nm).

Physicochemical data of 6a: $[\alpha]^{23}_D +27.0^\circ$ (c 1.0, H_2O); IR (KBr) ν_{max} 3404, 3323, 1705, 1246, 1057 cm^{-1} ; 1H NMR (D_2O , 400 MHz) δ 7.39–7.48 (5H, m, Ph), 5.12–5.17 (2H, m, OCH_2Ph), 4.20 (1H, m, H-3), 3.72 (1H, m, H-6a), 3.59 (1H, m, H-6b), 2.18 (1H, m, H-4a), 1.75–2.00 (3H, m, H-4b and H-5); ^{13}C NMR (D_2O , 100 MHz) δ 178.5 (C-2), 160.8 (NHCOO), 139.2 (Ph), 131.7 (Ph \times 2), 131.3 (Ph), 130.6 (Ph \times 2), 70.0 (OCH_2Ph), 55.0 (C-3), 48.0 (C-6), 29.1 (C-4), 23.9 (C-5); HRESIMS m/z 451.0432 $[M + Na]^+$ (calcd for $C_{13}H_{18}N_4Na_2O_7PS$, 451.0429); reversed-phase HPLC analysis, retention time 12.6 min (conditions were similar to those described for compound **5a**).

(3*R*,*R*_P)-1-Amino(sulfoamino)phosphinyl-3-benzyloxy-carbonylamino-2-piperidinone Sodium Salt (5b) and (3*R*,*S*_P)-1-Amino(sulfoamino)phosphinyl-3-benzyloxy-carbonylamino-2-piperidinone Sodium Salt (6b). The synthetic method was similar to that noted for compounds **5a** and **6a**. Compound **4b** (1.20 g, 3.68 mmol) was treated with SO_3 -pyridine complex (1.17 g, 7.35 mmol), followed by $NaHCO_3$ (0.77 g, 7.35 mmol), and the resultant diastereomer was separated to give **5b** (0.176 g) in 11% yield and **6b** (0.195 g) in

12% yield. To prepare analytical specimens of **5b** and **6b**, each optically pure (*R*)-(+)-1-phenylethylamine salt, which was purified in the next step, was changed into the sodium salt with sodium hydrogen carbonate and then purified by Diaion HP-20SS column chromatography (water–30% MeOH/water). The physicochemical data were obtained by using each resultant specimen.

Physicochemical data of 5b: $[\alpha]^{23}_D -25.3^\circ$ (c 0.5, H_2O); IR (KBr) ν_{max} 3408, 3294, 1705, 1248, 1057 cm^{-1} ; 1H NMR (D_2O , 400 MHz) δ 7.38–7.48 (5H, m, Ph), 5.12–5.17 (2H, m, OCH_2Ph), 4.20 (1H, m, H-3), 3.71 (1H, m, H-6a), 3.59 (1H, m, H-6b), 2.19 (1H, m, H-4a), 1.77–2.03 (3H, m, H-4b and H-5); ^{13}C NMR (D_2O , 100 MHz) δ 178.6 (C-2), 160.8 (NHCOO), 139.3 (Ph), 131.7 (Ph \times 2), 131.3 (Ph), 130.7 (Ph \times 2), 70.1 (OCH_2Ph), 55.1 (C-3), 48.1 (C-6), 29.2 (C-4), 24.0 (C-5); HRESIMS m/z 451.0428 $[M + Na]^+$ (calcd for $C_{13}H_{18}N_4Na_2O_7PS$, 451.0429); reversed-phase HPLC analysis, retention time 12.7 min (conditions were similar to those described for compound **5a**).

Physicochemical data of 6b: $[\alpha]^{23}_D +63.3^\circ$ (c 0.5, H_2O); IR (KBr) ν_{max} 3384, 3303, 1720, 1647, 1217, 1039 cm^{-1} ; 1H NMR (D_2O , 400 MHz) δ 7.39–7.49 (5H, m, Ph), 5.11–5.21 (2H, m, OCH_2Ph), 4.24 (1H, dd, $J = 11.2, 6.8$ Hz, H-3), 3.78 (1H, m, H-6a), 3.58 (1H, m, H-6b), 2.17 (1H, m, H-4a), 1.98 (1H, m, H-5a), 1.86 (1H, m, H-5b), 1.73 (1H, tdd, $J = 12.2, 8.8, 6.8$ Hz, H-4b); ^{13}C NMR (D_2O , 100 MHz) δ 178.0 (C-2), 161.0 (NHCOO), 139.3 (Ph), 131.7 (Ph \times 2), 131.3 (Ph), 130.6 (Ph \times 2), 70.0 (OCH_2Ph), 54.9 (C-3), 47.2 (C-6), 28.8 (C-4), 23.4 (C-5); HRESIMS m/z 451.0413 $[M + Na]^+$ (calcd for $C_{13}H_{18}N_4Na_2O_7PS$, 451.0429); reversed-phase HPLC analysis, retention time 14.0 min (conditions were similar to those described for compound **5a**).

(3*S*,*R*_P)-3-Amino-1-amino(sulfoamino)phosphinyl-2-piperidinone (7a). A mixture of (*S*)-(–)-1-phenylethylamine hydrochloride (0.40 g, 2.53 mmol) and compound **5a** (90% de, 0.80 g, 1.87 mmol) in water (4.0 mL) was applied to a Diaion HP-20SS (200 mL) column. The column was eluted with water–70% MeOH/water at room temperature, and the (*S*)-(–)-1-phenylethylamine salt of **5a** was collected in the fractions of 45–60% MeOH/water. The fractions including the ammonium salt of **5a** were combined and then evaporated under reduced pressure. The residue was applied to a Diaion HP-20SS (200 mL) column once more. After evaporation of the fractions under reduced pressure, the residue was dissolved in MeOH (10 mL) and was divided into 6 portions. Each portion was applied to a Sephadex LH-20 (200 mL) column, which was developed with MeOH. Then, the fractions of insufficient optical purity were combined and repeatedly subjected to similar column chromatography. The delayed fractions containing pure material (>99% de) were combined and evaporated under reduced pressure to give the optically pure (*S*)-(–)-1-phenylethylamine salt of **5a** (0.88 g).¹⁹ To a solution of the (*S*)-(–)-1-phenylethylamine salt of **5a** (0.88 g, 1.67 mmol) in MeOH (18.0 mL) and water (2.0 mL) was added Pd-black (0.18 g), and the suspension was stirred for 10 h at room temperature under hydrogen atmosphere. After the Pd-black was removed by filtration, the filtrate was evaporated under reduced pressure to afford a residue, which was purified by Diaion HP-20SS column chromatography (water). The solid residue was recrystallized from water/EtOH to give **7a** (0.35 g) in 65% yield: mp 203–208 °C (dec); $[\alpha]^{23}_D -21.5^\circ$ (c 1.0, H_2O); IR (KBr) ν_{max} 3510, 3355, 3250, 1658, 1545, 1308, 1188 cm^{-1} ; 1H NMR (D_2O , 500 MHz) δ 4.18 (1H, dd, $J = 12.0, 6.8$ Hz, H-3), 3.82 (1H, tdd, $J = 5.1, 13.0, 7.3$ Hz, H-6a), 3.70 (1H, tdd, $J = 5.1, 13.0, 6.7$ Hz, H-6b), 2.42 (1H, m, H-4a), 2.14 (1H, m, H-5a), 1.89–2.03 (2H, m, H-4b and H-5b); ^{13}C NMR (D_2O , 125 MHz) δ 172.4 (C-2), 51.3 (C-3), 45.4 (C-6), 24.2 (C-4), 20.5 (C-5); ^{31}P NMR (D_2O , 202 MHz) δ 6.03; HRESIMS m/z 271.0278 $[M - H]^-$ (calcd for $C_5H_{12}N_4O_5PS$, 271.0266); anal. C 20.41%, H 4.96%, N 19.44%, calcd for $C_5H_{13}N_4O_5PS \cdot H_2O$, C 20.69%, H 5.21%, N 19.30%.

(3*S*,*S*_P)-3-Amino-1-amino(sulfoamino)phosphinyl-2-piperidinone (8a). The synthetic method was similar to that described for compound **7a**. A mixture of (*S*)-(–)-1-phenylethylamine hydrochloride (0.58 g, 3.68 mmol) and compound **6a** (90% de, 1.04 g, 2.43 mmol) was purified to give the optically

pure (*S*)-(–)-1-phenylethylamine salt of **6a** (0.81 g).²⁰ The (*S*)-(–)-1-phenylethylamine salt of **6a** (0.81 g, 1.54 mmol) was treated with Pd-black (0.080 g) under hydrogen atmosphere. After the Pd-black was removed by filtration, the filtrate was purified by Diaion HP-20SS column chromatography and was recrystallized from water/EtOH to give **8a** (0.27 g) in 38% yield: mp 210–214 °C (dec); $[\alpha]_{\text{D}}^{25} +43.8^\circ$ (*c* 1.0, H₂O); IR (KBr) ν_{max} 3612, 3382, 3300, 1664, 1498, 1308, 1192 cm⁻¹; ¹H NMR (D₂O, 500 MHz) δ 4.15 (1H, dd, *J* = 11.7, 6.8 Hz, H-3), 3.62–3.77 (2H, m, H-6), 2.43 (1H, td, *J* = 12.2, 5.4 Hz, H-4a), 1.98–2.12 (2H, m, H-5), 1.93 (1H, tdd, *J* = 12.2, 9.5, 6.7 Hz, H-4b); ¹³C NMR (D₂O, 125 MHz) δ 172.4 (C-2), 51.3 (C-3), 45.7 (C-6), 24.6 (C-4), 20.9 (C-5); ³¹P NMR (D₂O, 202 MHz) δ 6.31; HRESIMS *m/z* 271.0277 [M – H][–] (calcd for C₅H₁₂N₄O₅PS, 271.0266); *anal.* C 20.79%, H 5.10%, N 19.59%, calcd for C₅H₁₃N₄O₅PS·H₂O, C 20.69%, H 5.21%, N 19.30%.

(3*R*,*R*_p)-3-Amino-1-amino(sulfoamino)phosphinyl-2-piperidinone (7b). The synthetic method was similar to that described for compound **7a**. A mixture of (*R*)-(+)-1-phenylethylamine hydrochloride (176.0 mg, 1.116 mmol) and compound **5b** (90% de, 176.0 mg, 0.411 mmol) was purified to give the optically pure (*R*)-(+)-1-phenylethylamine salt of **5b** (153.0 mg). The (*R*)-(+)-1-phenylethylamine salt of **5b** (145.0 mg, 0.275 mmol) was treated with Pd-black (14.5 mg) under hydrogen atmosphere. After the Pd-black was removed by filtration, the filtrate was purified by Diaion HP-20SS column chromatography and was recrystallized from water/EtOH to give **7b** (41.5 mg) in 37% yield: mp 208–211 °C (dec); $[\alpha]_{\text{D}}^{25} -43.6^\circ$ (*c* 0.5, H₂O); IR (KBr) ν_{max} 3610, 3386, 3300, 1664, 1498, 1308, 1192 cm⁻¹; ¹H NMR (D₂O, 500 MHz) δ 4.13 (1H, dd, *J* = 11.7, 6.8 Hz, H-3), 3.67–3.72 (2H, m, H-6), 2.42 (1H, td, *J* = 12.2, 5.3 Hz, H-4a), 1.96–2.11 (2H, m, H-5), 1.90 (1H, tdd, *J* = 12.2, 9.3, 6.4 Hz, H-4b); ¹³C NMR (D₂O, 125 MHz) δ 172.4 (C-2), 51.3 (C-3), 45.7 (C-6), 24.6 (C-4), 20.9 (C-5); ³¹P NMR (D₂O, 202 MHz) δ 6.29; HRESIMS *m/z* 271.0288 [M – H][–] (calcd for C₅H₁₂N₄O₅PS, 271.0266); *anal.* C 20.40%, H 5.03%, N 19.42%, calcd for C₅H₁₃N₄O₅PS·H₂O, C 20.69%, H 5.21%, N 19.30%.

(3*R*,*S*_p)-3-Amino-1-amino(sulfoamino)phosphinyl-2-piperidinone (8b). The synthetic method was similar to that described for compound **7a**. A mixture of (*R*)-(+)-1-phenylethylamine hydrochloride (200.0 mg, 1.269 mmol) and compound **6b** (90% de, 195.0 mg, 0.456 mmol) was purified to give the optically pure (*R*)-(+)-1-phenylethylamine salt of **6b** (174.5 mg). The (*R*)-(+)-1-phenylethylamine salt of **6b** (161.5 mg, 0.306 mmol) was treated with Pd-black (16.0 mg) under hydrogen atmosphere. After the Pd-black was removed by filtration, the filtrate was purified by Diaion HP-20SS column chromatography and was recrystallized from water/EtOH to give **8b** (74.6 mg) in 61% yield: mp 203–209 °C (dec); $[\alpha]_{\text{D}}^{25} +21.5^\circ$ (*c* 0.5, H₂O); IR (KBr) ν_{max} 3518, 3400, 3255, 1658, 1545, 1306, 1190 cm⁻¹; ¹H NMR (D₂O, 500 MHz) δ 4.17 (1H, dd, *J* = 11.7, 6.8 Hz, H-3), 3.81 (1H, tdd, *J* = 4.9, 12.7, 7.3 Hz, H-6a), 3.68 (1H, tdd, *J* = 4.9, 12.7, 6.8 Hz, H-6b), 2.40 (1H, m, H-4a), 2.14 (1H, m, H-5a), 1.87–2.02 (2H, m, H-4b and H-5b); ¹³C NMR (D₂O, 125 MHz) δ 172.4 (C-2), 51.3 (C-3), 45.4 (C-6), 24.2 (C-4), 20.5 (C-5); ³¹P NMR (D₂O, 202 MHz) δ 6.01; HRESIMS *m/z* 271.0273 [M – H][–] (calcd for C₅H₁₂N₄O₅PS, 271.0266); *anal.* C 20.41%, H 5.10%, N 19.26%, calcd for C₅H₁₃N₄O₅PS·H₂O, C 20.69%, H 5.21%, N 19.30%.

In Vitro Assay for DPP-IV Inhibitory Activity. Tris-(hydroxymethyl)aminomethane/maleic acid buffer solution (0.1 M, pH 7.2, 100 μL), 3.2 mM Gly-Pro-β-naphthylamide (25 μL, Bachem, Switzerland), and an aqueous solution (50 μL) of the synthetic compound were added into 96-well microplates. The resultant solution was warmed for 10 min at 37 °C, and then the DPP-IV solution (25 μL) was added to the solution. The combined solution was allowed to react for 1 h at 37 °C. The reaction was terminated by adding a solution (100 μL) of 0.2% Fast Garnet GBC salt (Sigma) in 0.5 M sodium citrate buffer solution (pH 3.78) including 10% polyoxyethylene (20) sorbitan monolaurate (Wako, Japan). The absorbance at 525 nm was measured (value *a*). Simultaneously, the absorbance of the reaction mixture without the synthetic compound solution was measured (value *b*). Moreover, the absorbance of the reaction

mixture without the DPP-IV solution was measured, respectively (value *a'* and value *b'*). The DPP-IV inhibitory rate (%) was calculated by $[(b - b') - (a - a')/(b - b')] \times 100$. From the inhibitory curve, taking inhibitory rate (%) at various concentrations of the synthetic compound on the ordinate and the logarithm of concentrations of the synthetic compound on the abscissa, the concentration for 50% inhibition was obtained. In all cases, a linear relation was observed between 20% and 80% inhibition. For example, the inhibitory rate of compound **7a** against 0.5, 1.0, 2.0, 4.0, 8.0, 16.0, 32.0, and 64.0 ng/mL was 15.9, 20.2, 30.6, 41.9, 54.4, 66.9, 77.4, and 84.5%, respectively. At this time, the IC₅₀ value of **7a** was 6.1 ng/mL. Furthermore, the IC₅₀ value of **7a** was measured 5 times, affording values of 5.8, 6.1, 6.3, 5.5, and 6.1 ng/mL, respectively. The concentrations of compounds **7b**, **8a**, and **8b** for 50% inhibition were obtained by a similar method, and this procedure was repeated 3 times or more. Then, each average was shown as the IC₅₀ value of the synthetic compound.

X-ray Diffraction Analysis. Compound 7a. A single crystal was obtained from a solution of water/ethanol. Crystal data for **7a**: colorless prisms (0.12 mm × 0.13 mm × 0.20 mm); molecular formula C₅H₁₃N₄O₅PS·H₂O, formula weight 290.23, orthorhombic, space group *P*2₁2₁2₁, *a* = 9.805(1) Å, *b* = 16.427(2) Å, *c* = 7.261(1) Å, *V* = 1169.5(2) Å³, *Z* = 4, *D*_{calc} = 1.648 g/cm³, *F*₀₀₀ = 608.00, μ (Cu Kα) = 40.47 cm⁻¹, 2400 measured intensities, 1204 unique (*R*_{int} = 0.009). The intensity data were collected on a Rigaku AFC-7R diffractometer using graphite-monochromated Cu Kα (λ = 1.54178 Å) radiation and a rotating anode generator with the ω -2 θ scan technique to a maximum 2 θ of 130.1°. Cell constants were refined from 25 well-centered reflections in the range 57.8° < 2 θ < 59.8°. The structure was solved by direct methods using SIR92²¹ and refined by full-matrix least-squares refinement. The final least-squares cycle was based on 1204 unique reflections, *R* = 0.072, *R*_w = 0.115, and *R*₁ = 0.039.²² The absolute configuration of the molecule was determined on the basis of Flack's parameter, –0.040(33).

Compound 8a. A single crystal was obtained from a solution of water/2-propanol. Crystal data for **8a**: colorless prisms (0.10 mm × 0.11 mm × 0.20 mm); molecular formula C₅H₁₃N₄O₅PS·H₂O, formula weight 290.23, orthorhombic, space group *P*2₁2₁2₁, *a* = 9.9694(8) Å, *b* = 15.337(1) Å, *c* = 7.585(1) Å, *V* = 1159.7(2) Å³, *Z* = 4, *D*_{calc} = 1.662 g/cm³, *F*₀₀₀ = 608.00, μ (Cu Kα) = 40.82 cm⁻¹, 2600 measured intensities, 1163 unique (*R*_{int} = 0.033). The intensity data were collected on a Rigaku AFC-7R diffractometer using graphite-monochromated Cu Kα (λ = 1.54178 Å) radiation and a rotating anode generator with the ω -2 θ scan technique to a maximum 2 θ of 130.3°. Cell constants were refined from 25 well-centered reflections in the range 56.3° < 2 θ < 59.7°. The structure was solved by direct methods using SIR92²¹ and refined by full-matrix least-squares refinement. The final least-squares cycle was based on 1204 unique reflections, *R* = 0.041, *R*_w = 0.063, and *R*₁ = 0.040.²² The absolute configuration of the molecule was determined on the basis of Flack's parameter, –0.139(35).

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Supporting Information Available: This material is available free of charge via the Internet at <http://pubs.acs.org>.

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- (19) The structure of the (S)-(-)-1-phenylethylamine salt of **5a** was confirmed by ¹H NMR: (CD₃OD, 400 MHz) δ 7.26–7.45 (10H, m, Ph×2), 5.09 (2H, s, OCH₂Ph), 4.47 (1H, q, J = 6.8 Hz, PhCHNH₂), 4.26 (1H, dd, J = 11.2, 6.3 Hz, H-3), 3.86 (1H, m, H-6a), 3.58 (1H, m, H-6b), 2.04–2.19 (2H, m, H-4a and H-5a), 1.72–1.85 (2H, m, H-4 and H-5b), 1.62 (3H, d, J = 6.8 Hz, CH₃).
- (20) The structure of the (S)-(-)-1-phenylethylamine salt of **6a** was confirmed by ¹H NMR: (CD₃OD, 400 MHz) δ 7.25–7.48 (10H, m, Ph×2), 5.07 (2H, br s, OCH₂Ph), 4.46 (1H, q, J = 6.8 Hz, PhCHNH₂), 4.22 (1H, dd, J = 11.2, 6.3 Hz, H-3), 3.77 (1H, m, H-6a), 3.63 (1H, m, H-6b), 2.11 (1H, m, H-4a), 1.83–2.00 (2H, m, H-5), 1.77 (1H, m, H-4b), 1.63 (3H, d, J = 6.8 Hz, CH₃).
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- (22) Crystallographic data for the structures reported in this paper have been deposited with the Cambridge Crystallographic Data Centre. Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44-(0)1223-336033 or e-mail: deposit@ccdc.cam.ac.uk).

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