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Discovery of long-acting *N*-(cyanomethyl)-*N*-alkyl-L-prolinamide inhibitors of dipeptidyl peptidase IV

Takashi Kondo,* Takahiro Nekado, Isamu Sugimoto, Kenya Ochi, Shigeyuki Takai, Atsushi Kinoshita, Akira Hatayama, Susumu Yamamoto, Kazuhito Kawabata, Hisao Nakai and Masaaki Toda

Minase Research Institute, Ono Pharmaceutical Co., Ltd, 3-1-1 Sakurai, Shimamoto, Mishima, Osaka 618-8585, Japan

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Abstract—Details of structure–activity relationships (SAR) for P2 moiety of a P1 2-cyanopyrrolidine dipeptidyl peptidase IV (DPP-IV) inhibitor **4a** including stereochemistry are presented. Based on this information, a series of P1 (*N*-alkyl)aminoacetonitrile analogs **9**–**20** possessing optimal P2 structure were synthesized and evaluated as inhibitors of DPP-IV. Among them, a representative compound **11**, *N*-(cyanomethyl)-*N*-ethyl-L-prolinamide, was further evaluated to determine its effect on the plasma glucose level. Also **4a**, **10**, and **11** were evaluated for their isozyme selectivity to predict their safety problems.

1. Introduction

Incretins are peptide hormones that are released by the gastrointestinal tract in response to digestion of food and stimulate insulin secretion. Enhanced incretin activity should lead to sustained insulin secretion, which in turn could normalize an elevated blood glucose level. For this reason, the incretin GLP-1 has received considerable attention.[1–7] It has been shown that intravenous infusion of GLP-1 almost normalizes the blood glucose level in type 2 diabetic patients.[8] However, due to its peptide nature and short half-life, GLP-1 is not suitable as an orally active medicine. An indirect way to increase the level of GLP-1 is to inhibit dipeptidyl peptidase IV (DPP-IV), which is the enzyme responsible for rapid degradation of GLP-1 in vivo.[9] Consequently, low molecular weight DPP-IV inhibitors look promising as potential new medications for the treatment of type 2 diabetes.[10–16] Recently, non-peptidic inhibitors **1**–**3** (Fig. 1) have been clinically developed. Among them, **2** was approved as a first-in-class drug. As illustrated by their PK profiles, safe and long-acting inhibitors have been clinically needed for good quality of life (QOL).

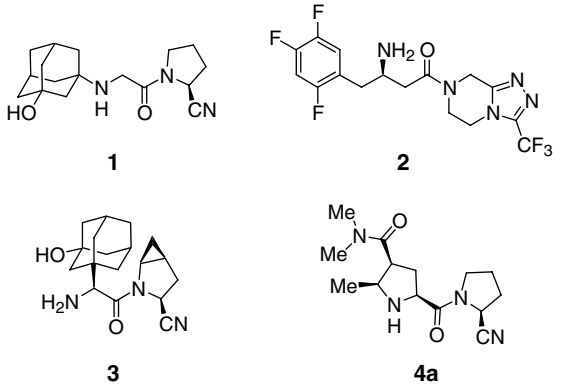

Figure 1. Long-acting inhibitors of DPP-IV.

Several DPP-IV inhibitors possessing modified proline structure as P2 moiety have been reported as illustrated below. In 2005, Sakashita et al. reported that (4-substituted)-L-prolyl-(2*S*)-2-cyanopyrrolidines showed increased inhibition of DPP-IV activity relative to unsubstituted analogs and that (4β-substituted)-L-prolyl-(2*S*)-2-cyanopyrrolidines showed 20-fold stronger activity than the corresponding 4-α isomer.[17] In addition, Tsai et al. reported that (4β-carbamoyl)-L-prolyl-(2*S*)-2-cyanopyrrolidines showed enhanced DPP-IV inhibitory activity, while (5,5-*gem*-dimethyl)-L-prolyl-(2*S*)-2-cyanopyrrolidine showed a 500-fold decrease of

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* Corresponding author. Tel.: +81 75 961 1151; fax: +81 75 962 9314; e-mail: t.kondou@ono.co.jp

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DPP-IV inhibition relative to the unsubstituted analog.[18] Pei et al. also reported (5-substituted-pyrrolidinyl-2-carbonyl)-2-cyanopyrrolidines as potent DPP-IV inhibitors.[19]

We previously reported on the discovery of a highly efficacious and long-acting proline-based inhibitor **4a** (Fig. 1).[20] Our effort was further continued to identify structurally new inhibitors possessing in vivo efficacy and long-acting PK profile without safety problems. Compound **4a** has 4,5-disubstituted proline moiety as a novel P2 structure. But it is not confirmed whether the P2 structure is optimized or not. One of the objectives of this work is to describe more detailed SAR study of P2 structure of **4a** including the stereochemistry before starting further molecular design based on this novel P2 structure. Using the newly identified optimal P2 structure, we found (*N*-alkyl)aminoacetonitrile as another P1 structure instead of 2-cyanopyrrolidine. Here we report the discovery process of long-acting DPP-IV inhibitors which consist of the newly identified optimal P2 structure and (*N*-alkyl)aminoacetonitrile P1 structure (Scheme 1).

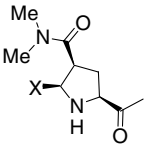
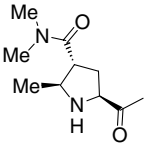
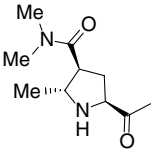
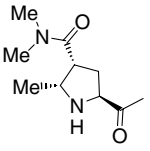
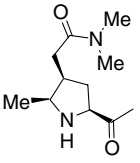
Another objective of this work is to disclose isozyme (DPP-IV, DPP-VIII, and DPP-IX) selectivity profiles of open-chain P1-based inhibitors for the safety concern.[21] Magnin et al. reported detailed in vitro SAR study and brief in vivo results of the related (*N*-alkyl)aminoacetonitrile-based inhibitors but they did not refer to the isozyme selectivity of their inhibitors.[22]

As a result, we identified *N*-(cyanomethyl)-*N*-ethyl-L-prolinamide as another highly efficacious, more isozyme-selective, and long-acting inhibitor of DPP-IV.

2. Chemistry

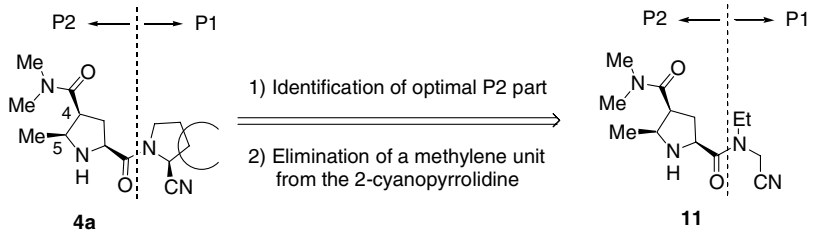
Synthesis of the compounds listed in Tables 1–3 is outlined in Schemes 2–7. The 5β-ethyl analog **4b** was synthesized from appropriately protected L-glutamic acid **21** as described in Scheme 2. Acylation of the γ-carbon of **21** with propionyl chloride, followed by acidic dehydration with trifluoroacetic acid, resulted in the formation of a cyclized product **22**. Catalytic hydrogenation of **22** in the presence of platinum oxide exclusively afforded the 2,4,5-*cis* isomer **23**. Acidic treatment of **23**, followed by N-protection with Boc_2O, led to **24**. A peptide formation reaction of **24** with L-prolinamide gave **25**, after which alkaline hydrolysis resulted in **26**.

Table 1. In vitro inhibition for human DPP-IV and ex vivo plasma DPP-IV inhibition in normal rats

Compound	R	Human DPP-IV IC_{50} (nM)	Plasma DPP-IV inhibition (%) at 0.3 mg/kg po, normal rats 6 h
			
4a	X = Me	10	89
4b	X = Et	16	45
5		1100	NT[a]
6		70	NT[a]
7		110	NT[a]
8		5.6	40

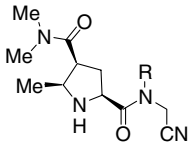
[a] Not tested.

Esterification of the carboxylic acid group of **26** with benzyl bromide in the presence of potassium carbonate afforded **27**. Dehydration of the primary amide of **27** followed by catalytic hydrogenation produced **29**. Amidation of **29** with *N*,*N*-dimethylamine, followed by acidic deprotection, yielded **4b** as a *p*-toluenesulfonate.



Scheme 1. Discovery process of *N*-(cyanomethyl)-*N*-ethyl-L-prolinamide inhibitor of DPP-IV.

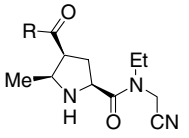
Table 2. In vitro inhibition for human DPP-IV and ex vivo plasma DPP-IV inhibition in normal rats

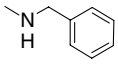


Compound	R	Human DPP-IV IC_{50} (nM)	Plasma DPP-IV inhibition (%) at 0.3 mg/kg po, normal rats 6 h
4a		10	89 (83)[a]
9	H	13,000	NT[b]
10	Me	25	55
11	Et	24	73 (62)[a]
12	^{n}Pr	600	NT[b]
13	allyl	41	19
14	cyclopropyl	56	29
15	propargyl	60	18

[a] Plasma DPP-IV inhibition (%) at 9 h after the oral dosing.
[b] Not tested.

Table 3. In vitro inhibition for human DPP-IV and ex vivo plasma DPP-IV inhibition in normal rats



Compound	R	Human DPP-IV IC_{50} (nM)	Plasma DPP-IV inhibition (%) at 0.3 mg/kg po, normal rats 6 h
11	NMe_2	24	73
			
16	$n = 2$	16	53
17	$n = 3$	17	60
18		24	65
19		11	64
20		51	31

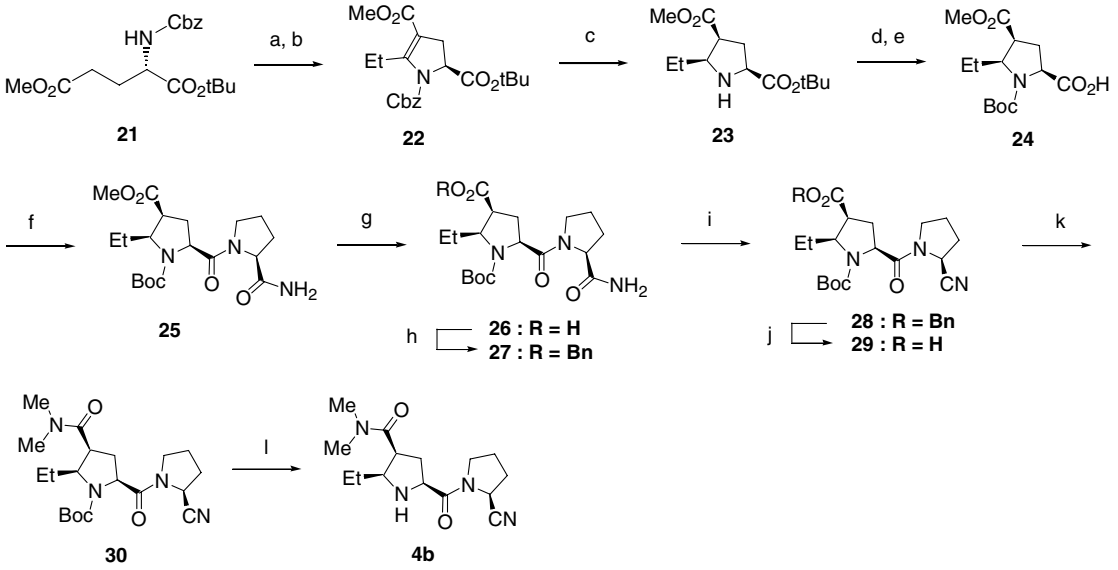
Synthesis of **5** is outlined in Scheme 3. Catalytic hydrogenation of **31**[20] in the presence of palladium–carbon resulted in the formation of an isomeric mixture of **32a** and **32b** (ratio = 3:2). The stereochemical result (**32a**:**32b** = 3:2) was derived from enamineimine equilibration followed by the reduction of isomeric mixture of imine forms after the removal of *N*-benzyloxycarbonyl group of **31**. Acidic deprotection of **32b**, followed by N-protection with a Boc group, resulted in **33**. Amidation of **33** with L-prolinamide afforded **34**, alkaline hydrolysis of which led to the corresponding carboxylic acid **35**. Dehydrative condensation of **35** with *N*,*N*-dimethylamine provided *N*,*N*-dimethylamide **36**, after which further dehydration resulted in formation of the corresponding nitrile **37**. Acidic deprotection of **37** produced the 4α-amide isomer **5**.

Synthesis of **6**–**7** is outlined in Scheme 4. Alkylation of the γ-carbon of N-protected ethyl L-pyroglutamate **38** with benzyloxymethyl chloride afforded a mixture of diastereomers **39a** and **39b** (ratio = 3:2). Partial reduction of the cyclic imide carbonyl of **39a**, followed by acetalization, gave the methyl acetal **40**. Stereoselective 5α-methylation of **40** with methyl Grignard reagent and copper(I) bromide–dimethylsulfide complex in the presence of boron trifluoride-etherate, followed by protection of the deprotected nitrogen, led to **41**, catalytic hydrogenolysis of which provided the alcohol **42**. Jones oxidation of **42** resulted in carboxylic acid **43**, condensation of which with *N*,*N*-dimethylamine produced **44**. Alkaline hydrolysis of **44** afforded a carboxylic acid **45**, after which peptide formation with (2*S*)-2-cyanopyrrolidine provided **46**. Acidic deprotection of **46** led to production of the 5α-diastereoisomer **6**. Note that **39b** was converted to the corresponding 4α,5α-diastereoisomer **7** by essentially same reaction sequences as described above.

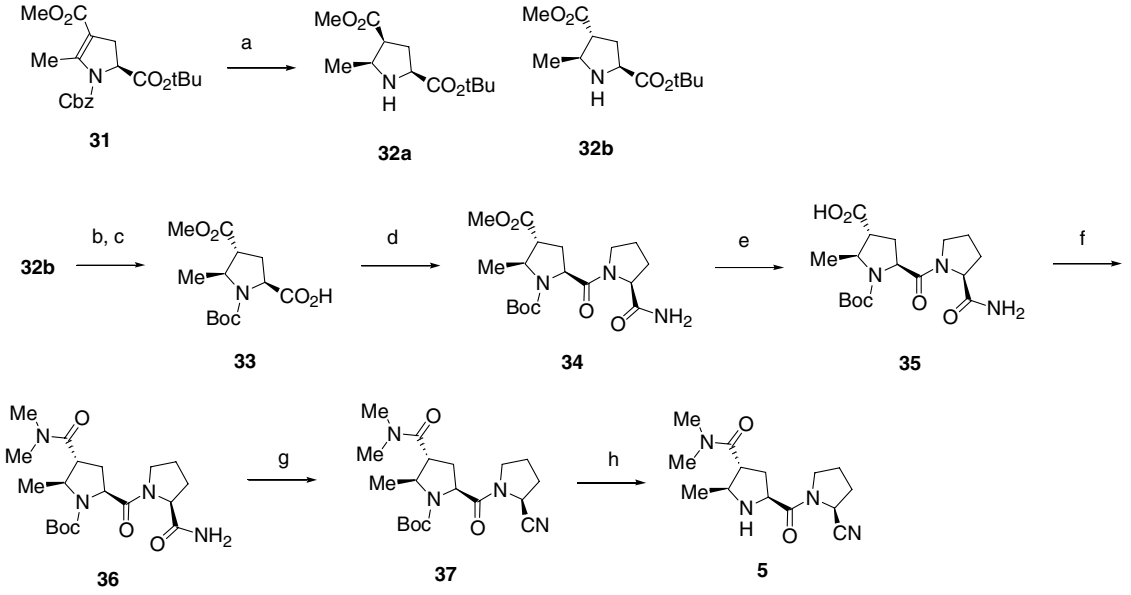
Synthesis of the 4β-acetoamide analog **8** is described in Scheme 5. Homologation of the 4β-carboxylic acid group of **54**[20] was carried out according to the conventional Arndt–Eistert reaction, resulting in the formation of **56**, alkaline hydrolysis of which provided the carboxylic acid **57**. Standard amidation of **57** with *N*,*N*-dimethylamine afforded **58**. Dehydration of the amide group of **58** produced a nitrile **59**, deprotection of which led to the amide nitrile **8**.

Synthesis of **9**–**15** is outlined in Scheme 6. Acetylation of the γ-carbon of an appropriately protected L-glutamic acid **60**, followed by treatment with an acid, afforded a cyclic product **61**, after which selective hydrogenation provided a carboxylic acid **62**. Dehydrative condensation of **62** with *N*,*N*-dimethylamine gave an amide **63**, hydrogenation of which in the presence of platinum oxide led to stereoselective production of the 2,4,5-*cis*-product **64**. Catalytic hydrogenation of **63**, which is protected with *tert*-butyloxycarbonyl group, gave a single diastereomer because of no enamine–imine equilibration as described in the hydrogenation of **31**. Alkaline hydrolysis of **64** afforded the corresponding carboxylic acid **65**, which was used as a key intermediate for further transformation. Acidic deprotection of **65** was followed by N-protection with benzyloxycarbonyl to afford **66**. Treatment of **66** with oxalyl chloride followed by *N*-methylglycinamide produced **67**. Deprotection of **67** was followed by N-protection with the *tert*-butyloxycarbonyl group to afford **68**, dehydration of which led to the nitrile **69b**. Acidic deprotection of **69b** gave **10**. Treatment of **65** with cyanuric fluoride in pyridine, followed by aminoacetonitrile and appropriate (*N*-alkyl)aminoacetonitriles, resulted in **69a** and **69c**–**g**, respectively. Acidic deprotection of **69a** and **69c**–**g** gave **9** and **11**–15, respectively.

Synthesis of**16**–**20** is shown in Scheme 7. Acetylation of the γ-carbon of an appropriately protected L-glutamic



Scheme 2. Synthesis of **4b**. Reagents: (a) EtCOCl, LiHMDS, THF; (b) TFA, CH_2Cl_2; (c) H_2, PtO_2, AcOH; (d) TFA aq; (e) Boc_2O, $NaHCO_3$ aq, THF; (f) L-ProNH_2, EDC, HOBt, Et_3N, CH_2Cl_2; (g) LiOH aq, MeOH; (h) BnBr, K_2CO_3, DMF; (i) TFAA, pyridine, THF; (j) H_2, $Pd(OH)_2$, EtOAc, THF; (k) Me_2NH, EDC, HOBt, Et_3N, CH_2Cl_2; (l) *p*-TsOH, EtOH.

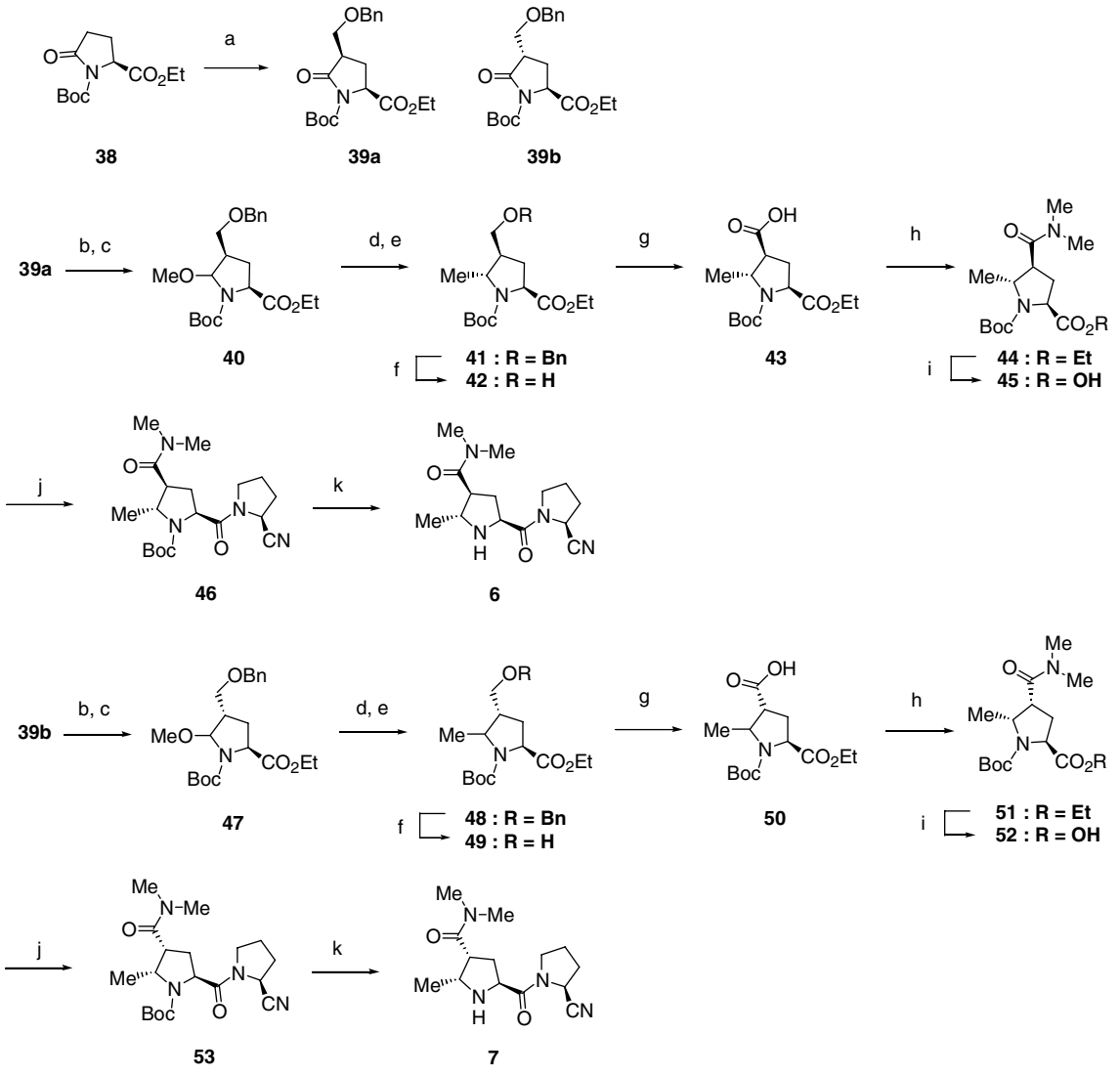


Scheme 3. Synthesis of **5**. Reagents: (a) H_2, 10% Pd/C, AcOH; (b) TFA aq; (c) Boc_2O, $NaHCO_3$ aq, THF; (d) L-ProNH_2, EDC, HOBt, Et_3N, CH_2Cl_2; (e) NaOH aq, MeOH; (f) Me_2NH, EDC, HOBt, Et_3N, CH_2Cl_2; (g) TFAA, pyridine, THF; (h) 4 N HCl/EtOAc.

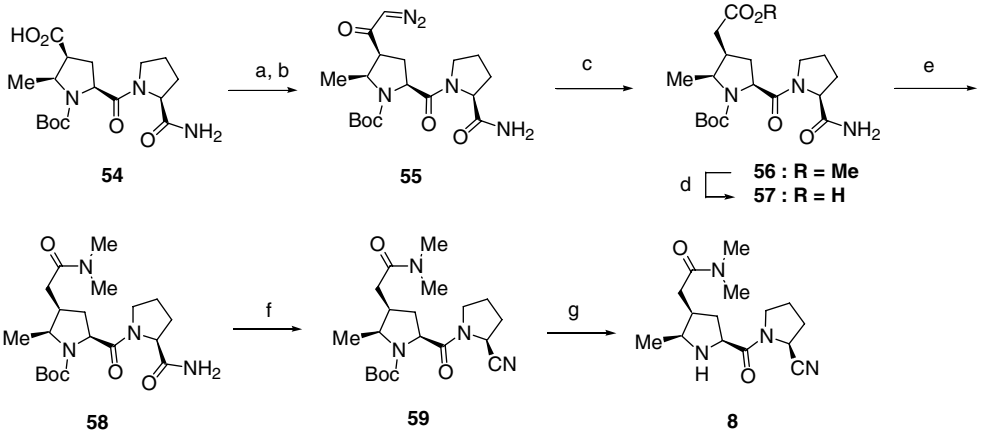
acid **70** was followed by treatment with an acid to afford **71**, catalytic hydrogenation of which in the presence of palladium–carbon provided **72**. Peptidation of **72** with *N*-ethylglycinamide in the presence of cyanuric fluoride in pyridine provided **73**, after which catalytic hydrogenation exclusively produced the 2,4,5-*cis*-isomer **74**. Catalytic hydrogenation of **73** also resulted in the exclusive production of a single isomer because of the same reason as described in catalytic hydrogenation of **63**. Alkaline hydrolysis of **74** was followed by *O*-benzylation to provide **76**, dehydration of which gave a nitrile **77**. Deprotection of **77** was successfully carried out by catalytic hydrogenation in the presence of palladium hydroxide, resulting in **78**. Condensation of **78** with appropriate amines in the presence of the polystyrene carbodiimide (PS-carbodiimide) and polystyrene *N*-methylmorpholine (PS-NMM) led to production of **79a**–**e**, respectively. Acidic deprotection of **79a**–**e** afforded **16**–**20**.

3. Results and discussion

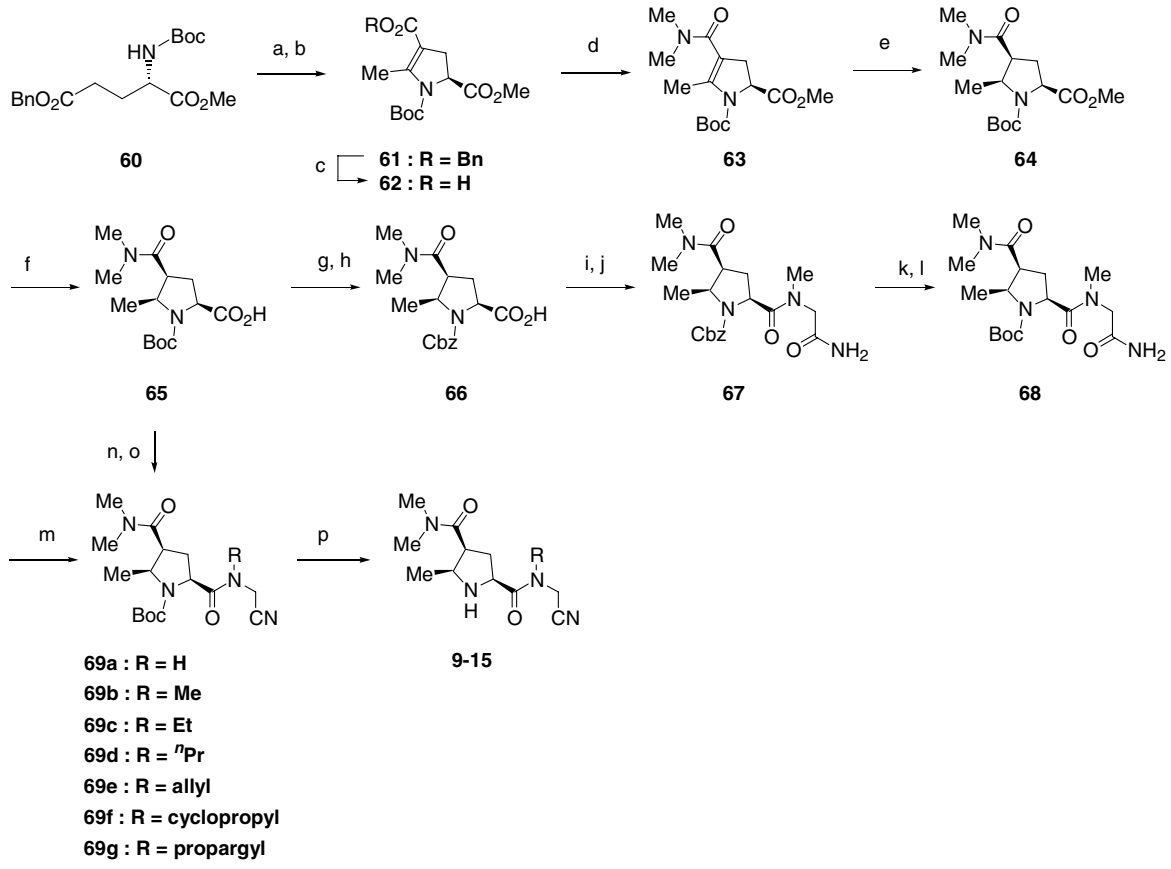
All of the compounds listed in Tables 1–3 were tested in vitro using purified human DPP-IV enzyme to assess inhibition of its metabolism of the synthetic substrate H-Gly-Pro-AMC.[23,24] Production of 7-amino-4-methyl coumarin (AMC) was measured over 15 min at 460 nm. Plasma DPP-IV inhibition (%) by the test compounds after oral administration (0.3 mg/kg) was monitored over



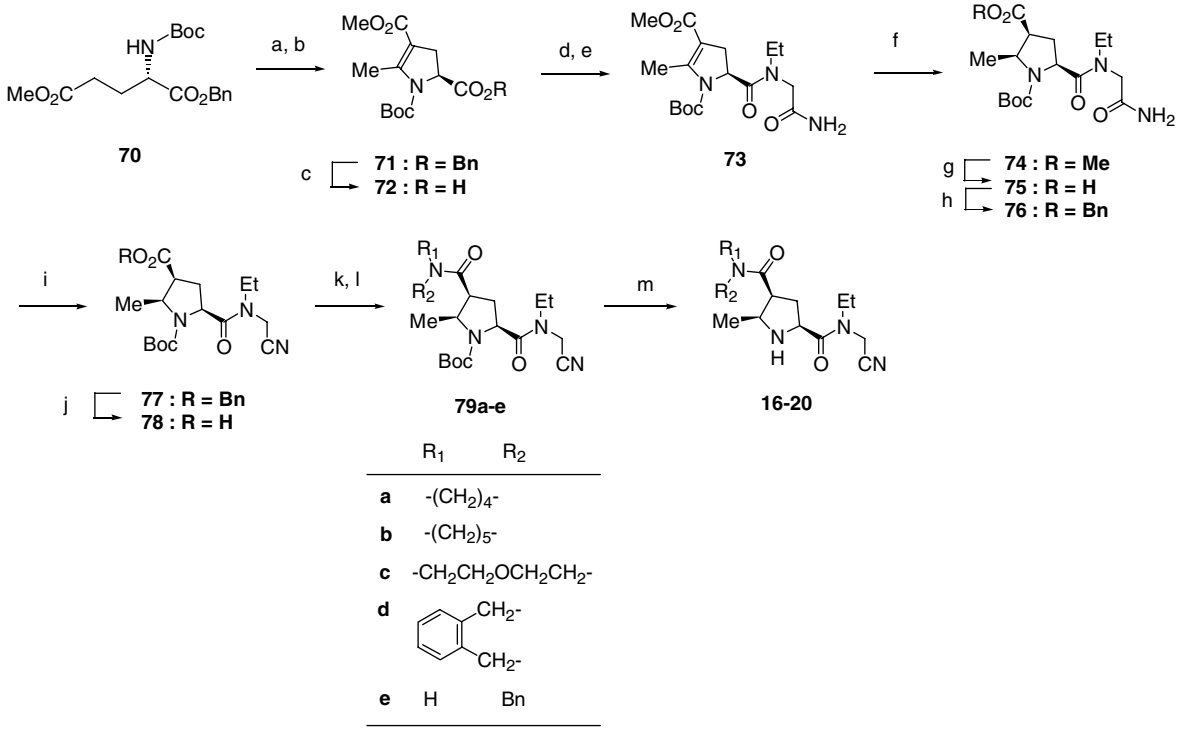
Scheme 4. Synthesis of **6**–**7**. Reagents: (a) LiHMDS, $BnOCH_2Cl$, HMPA, THF; (b) $LiBEt_3H$, THF; (c) *p*-TsOH, MeOH; (d) MeMgBr, CuBr–Me_2S, BF_3–OEt_2, Et_2O; (e) Boc_2O, $NaHCO_3$ aq, THF; (f) H_2, 10% Pd/C, EtOH; (g) Jones reagent, acetone; (h) Me_2NH, EDC, HOBt, Et_3N, CH_2Cl_2; (i) NaOH aq, MeOH; (j) L-ProCN, EDC, HOBt, Et_3N, CH_2Cl_2; (k) *p*-TsOH, EtOH.



Scheme 5. Synthesis of **8**. Reagents: (a) $ClCO_2Et$, Et_3N, THF; (b) CH_2N_2, Et_2O, THF; (c) BzOAg, Et_3N, MeOH; (d) NaOH aq, MeOH; (e) Me_2NH, EDC, HOBt, Et_3N, CH_2Cl_2; (f) TFAA, pyridine, THF; (g) *p*-TsOH, EtOH.



Scheme 6. Synthesis of **9**–**15**. Reagents: (a) Ac_2O, LiHMDS, THF; (b) TFA, CH_2Cl_2; (c) H_2, 10% Pd/C, MeOH; (d) Me_2NH, EDC, HOBt, NMM, CH_2Cl_2; (e) H_2, PtO_2, AcOH; (f) NaOH aq, THF; (g) 4 N HCl/1,4-dioxane, CH_2Cl_2; (h) CbzCl, $NaHCO_3$ aq, THF; (i) $(COCl)_2$, DMF, CH_2Cl_2; (j) $MeNHCH_2CONH_2$, Et_3N, CH_2Cl_2; (k) H_2, Pd/C, THF; (l) Boc_2O, THF; (m) TFAA, pyridine, CH_2Cl_2; (n) cyanuric fluoride, pyridine, CH_2Cl_2; (o) $RNHCH_2CN$, pyridine, $ClCH_2CH_2Cl$; (p) *p*-TsOH, EtOH.



	R_1	R_2
a	$-(CH_2)_4-$	
b	$-(CH_2)_5-$	
c	$-CH_2CH_2OCH_2CH_2-$	
d	$-CH_2$	$-CH_2$
e	H	Bn

Scheme 7. Synthesis of **16**–**20**. Reagents: (a) Ac_2O, LiHMDS, THF; (b) TFA, CH_2Cl_2; (c) H_2, 10% Pd/C, MeOH; (d) cyanuric fluoride, pyridine, CH_2Cl_2; (e) $EtNHCH_2CN$, pyridine, $ClCH_2CH_2Cl$; (f) H_2, PtO_2, AcOH; (g) LiOH aq, MeOH; (h) BnBr, K_2CO_3, DMF; (i) TFAA, pyridine, THF; (j) H_2, $Pd(OH)_2$, EtOAc; (k) PS-carbodiimide, HOBt, CH_2Cl_2; (l) R_1R_2NH, PS-NMM, CH_2Cl_2; (m) *p*-TsOH, ^{i}PrOH.

a 6-h period in normal rats. The % inhibition at 6 h after oral dosing is shown as an index of the duration of action.

Before starting further molecular design of P1 (*N*-alkyl)aminoacetonitrile analogs, it was necessary to confirm whether the P2 structure of **4a** is already optimized or not.

To discuss more detailed SAR of P2 structure of **4a**, including the stereochemistry, synthesis and biological evaluation of the stereoisomers **5**–**7** was carried out as shown in Table 1. All of the stereoisomers **5**–**7** exhibited a decrease of inhibitory activity. As a result, the stereochemistry of **4a** (4*S*,5*S*) was found to be the most optimal among those tested. Keeping these SAR data in mind, further efforts to optimize 4- and 5-substituents were continued. Replacement of the 5β-methyl group of **4a** with a 5β-ethyl group provided **4b**, which had slightly less potent inhibitory activity and a shorter duration of ex vivo activity. Replacement of the 4β-*N*,*N*-dimethylaminocarbonyl group of **4a** with the 4β-*N*,*N*-dimethylaminocarbonylmethyl group afforded **8**, which retained in vitro inhibitory activity and showed a shorter duration of plasma DPP-IV inhibition.

Thus, the stereochemistry and the 4-, 5-substituents of **4a** were found to be the most optimal as a P2 moiety within the SAR tested. The marked reduction of the inhibitory activity of (5,5-*gem*-dimethyl)-L-prolyl-(2*S*)-2-cyanopyrrolidine as reported by Tsai et al. was considered to be mainly due to the undesirable 5α-methyl group, which was speculated to prevent the analog from interacting with the enzyme.[18]

Next, attention was paid to chemical modifications of the right half (P1 moiety) of **4a** as illustrated in Table 2. The aminoacetonitrile and (*N*-alkyl)aminoacetonitrile analogs **9**–**15** were designed based on the concept described in Scheme 1, followed by synthesis and evaluation. Replacement of the (*S*)-2-cyanopyrrolidine moiety of **4a** with a glycine moiety afforded **9**, which showed a marked decrease of inhibitory activity, while the corresponding *N*-methyl and *N*-ethyl analogs **10**–**11** showed slightly less potent inhibition and a shorter duration of action relative to **4a**. The *N*-ethyl analog **11** demonstrated more potent plasma DPP-IV inhibition than the *N*-methyl analog **10** at 6 h after oral dosing. The *N*-*n*-propyl analog **12** showed a marked decrease of in vitro activity, while the *N*-allyl, *N*-cyclopropyl, and *N*-propargyl analogs **13**–**15** showed restoration of inhibitory activity, but achieved much weaker plasma DPP-IV inhibition after 6 h. Consequently, in vitro SAR of **9**–**14** demonstrated close to that of the corresponding BMS compounds.[22] As a result, the (*N*-ethyl)aminoacetonitrile analog **11** exhibited the most potent in vitro activity and the longest duration of ex vivo activity among this series of analogs **9**–**15**.

DPP-IV inhibition at 9 h after oral dosing, **11** showed 62% inhibition of DPP-IV, while **4a** showed 83% inhibition in a simultaneous experiment. Among the series of analogs listed in Table 2, compound **11** with the most similar molecular formula to **4a** showed the most desirable activity profile. The *N*-*n*-propyl analog **12** exhibited unexpectedly weak inhibitory activity compared with the other analogs **13**–**15**, but the reason is unclear.

As shown in Table 3, readjustment of the amide moiety of **11** was carried out. Most of the cyclic amide analogs **16**–**19** exhibited nearly equipotent in vitro activity and slightly weaker plasma DPP-IV inhibition at 6 h after oral dosing, while the *N*-benzylamide analog **20** showed weaker in vitro activity and obviously weaker ex vivo activity. Also in this series of analogs, the *N*,*N*-dimethylaminocarbonyl was found to be the most optimal partial structure as the 4β-substituent. The relatively longer duration of action shown by **4a** and **11** was speculated to be related to the presumed greater stability against liver metabolism of the 4β-substituent (*N*,*N*-dimethylaminocarbonyl), the clog*P* value of which was lower than those of the others tested in each series.

Isozyme selectivity was reported to be one of the important factors to predict the safety problems of these inhibitors.[21] Based on this information, we evaluated our inhibitors **4a** and **10**–**11** for their isozyme selectivity which Magnin et al. did not report.[22]

Isozyme selectivity of some representative compounds (**4a** and **10**–**11**) was investigated, as shown in Table 4, and all of these analogs were found to exhibit relatively high selectivity for DPP-VIII and DPP-IX.[25,26] In particular, analogs **10**–**11** showed greater isozyme selectivity than **4a**.

The new inhibitor **11**, bearing (*N*-ethyl)aminoacetonitrile P1 group, was evaluated to determine its effect on plasma glucose level. As displayed in Figure 2, the effects

Table 4. Selectivity of representative DPP-IV inhibitors

Compound	Human DPP-IV IC_{50} (nM)	Human DPP-VIII IC_{50} (nM)	Human DPP-IX IC_{50} (nM)
4a	10	14,000	2500
10	25	>100,000	58,000
11	24	>100,000	32,000

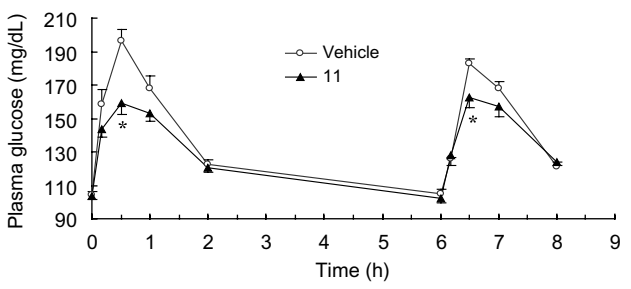


Figure 2. Effect of the inhibitor **11** on glucose excursion during multiple oral glucose tolerance tests in normal rats. All rats received 1 g/kg glucose orally at 0, 6 h. The compound (1 mg/kg) was orally administered to rats at −0.5 h. Data are expressed as means ± SEM ($n = 7$). $^{*}P < 0.05$; significantly different from the vehicle by Student's *t* test.

of inhibitor **11** (1 mg/kg po) and the vehicle control on the plasma glucose level after the oGTT were investigated in normal rats. Compound **11** was able to reduce plasma glucose even after 7 h, corresponding to its plasma DPP-IV inhibition.

In summary, P2 structure of **4a** was confirmed to be the most optimal structure including the stereochemistry among the tested compounds. Further SAR study of its P1 (*N*-alkyl)aminoacetonitrile analogs **9**–**20** possessing the optimal P2 structure resulted in the discovery of an open-chain P1-based inhibitor **11**. It showed the most potent in vitro activity and the longest duration of action among the series of analogs **9**–**15**, whose ex vivo duration of action was shorter than that of **4a**.[20] SAR study of the 4β-amido moiety of **11** was further conducted and 4β-*N*,*N*-dimethylaminocarbonyl group of **4a** was again confirmed to be the best one among the tested compounds **11** and **16**–**20** while **20** possessing *N*-benzylamide moiety showed less potent inhibitory activity and the shortest duration of action. Compounds **10** and **11** showed greater isozyme selectivity than **4a**. Compound **11** reduced the plasma glucose level over time in a manner which was consistent with its ex vivo plasma DPP-IV inhibition.

4. Experimental

4.1. Chemistry

Analytical samples were homogeneous as confirmed by thin layer chromatography (TLC) and afforded spectroscopic results consistent with the assigned structures. Proton nuclear magnetic resonance spectra (^{1}H NMR) were taken on a Varian Mercury 300 spectrometer using deuterated chloroform ($CDCl_3$) or deuterated dimethylsulfoxide (DMSO-d_6) as the solvent. The chemical shift values are reported in parts per million (δ) and coupling constants (J) in hertz (Hz). Fast atom bombardment mass spectra (FAB-MS, HRMS) and electron ionization (EI) were obtained on a JEOL JMS-700 spectrometer. Atmospheric pressure chemical ionization (APCI) was determined on a HITACHI M-1200H spectrometer. Infrared spectra (IR) were measured in a JASCO FT/IR-430 spectrometer. Column chromatography was carried out on silica gel [Merck silica gel 60 (0.063–0.200 mm), Wako gel C200 or Fuji Silysia FL60D]. TLC was performed on silica gel (Merck TLC or HPTLC plates, silica gel 60 F254). The following abbreviations for solvents and reagents are used; tetrahydrofuran (THF), diethyl ether (Et_2O), diisopropyl ether ($^i Pr_2O$), *tert*-butyl methyl ether (^{t}BuOMe), dimethylsulfoxide (DMSO), ethyl acetate (EtOAc), dimethylformamide (DMF), dichloromethane (CH_2Cl_2), chloroform ($CHCl_3$), methanol (MeOH), ethanol (EtOH), acetic acid (AcOH), hexamethylphosphoric triamide (HMPA), and hydrochloric acid (HCl).

4.1.1. 1-Benzyl 2-*tert*-butyl 4-methyl (2*S*)-5-ethyl-2,3-dihydro-1*H*-pyrrole-1,2,4-tricarboxylate (22). To a stirred solution of lithium bis(trimethylsilyl)amide in THF (100 mL, 1.0 M) was added dropwise a solution of **21** (14.0 g, 40 mmol) in THF (40 mL) at −78 °C. After being stirred for 30 min, the reaction mixture was treated with propionyl chloride (5.2 mL, 60 mmol) and stirred at −78 °C for additional 2 h. The reaction was quenched with 5% aqueous $KHSO_4$ and the mixture extracted with EtOAc. The organic layer was successively washed with aqueous $NaHCO_3$, brine, then dried over $MgSO_4$ and evaporated. To a stirred solution of the resulting residue in CH_2Cl_2 (40 mL) was added trifluoroacetic acid (3.1 mL, 40 mmol) at 0 °C. After being stirred for 2 h at room temperature, the reaction mixture was diluted with EtOAc and treated with 1 M NaOH. The organic layer was successively washed with aqueous $NaHCO_3$, brine, then dried over $MgSO_4$, and evaporated. The resulting residue was purified by silica gel chromatography using EtOAc/hexane (1:4) as an eluant to yield **22** (10.1 g, 65%) as a white powder. TLC $R_f = 0.78$ (EtOAc/hexane, 1:2); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.19 (t, $J = 7.5$ Hz, 3H), 1.36 (s, 9H), 2.67 (dd, $J = 15.9$, 3.9 Hz, 1H), 3.02–3.29 (m, 3H), 3.71 (s, 3H), 4.63 (dd, $J = 12.0$, 4.2 Hz, 1H), 5.16 (s, 2H), 7.30–7.38 (m, 5H).

4.1.2. (2*S*,4*S*,5*S*)-1-(*tert*-Butoxycarbonyl)-5-ethyl-4-(methoxycarbonyl)-2-pyrrolidinecarboxylic acid (24). To a solution of **22** (10.0 g, 25.7 mmol) in AcOH (50 mL) was added platinum(IV) oxide (1.0 g, 4.4 mmol). The mixture was vigorously stirred at room temperature under an atmospheric pressure of hydrogen for 8 h. The catalyst was removed by filtration and the filtrate was concentrated in vacuo. A solution of the resulting residue in trifluoroacetic acid (18 mL) and water (2 mL) was stirred at room temperature for 19 h. The reaction mixture was evaporated. To a stirred solution of the resulting residue in THF (3 mL) and water (13 mL) were added $NaHCO_3$ (10.8 g, 129 mmol) and a solution of di-*tert*-butyl-dicarbonate (6.72 g, 31 mmol) in THF (10 mL) at room temperature. After being stirred for 15 h, the reaction was quenched with 2 M HCl and the mixture extracted with EtOAc. The organic layer was washed with brine, dried over $MgSO_4$, and evaporated to yield **24** (3.60 g, 47%). TLC $R_f = 0.33$ (EtOAc); MS (APCI, pos. 20 V) *m*/*z* 302 $(M+H)^+$; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.92 (t, $J = 7.2$ Hz, 3H), 1.30–1.50 (m, 2H), 1.48 (s, 9H), 2.30–2.50 (m, 1H), 2.60–2.85 (m, 1H), 3.08–3.18 (m, 1H), 3.72 (s, 3H), 4.20–4.26 (m, 1H), 4.30–4.43 (m, 1H).

4.1.3. 1-*tert*-Butyl 3-methyl (2*S*,3*S*,5*S*)-5-{[(2*S*)-2-carbamoyl-1-pyrrolidinyl]carbonyl}-2-ethyl-1,3-pyrrolidinedicarboxylate (25). To a stirred solution of **24** (3.58 g, 11.9 mmol), in CH_2Cl_2 (12 mL) were added L-prolinamide (1.49 g, 13.1 mmol), 1-hydroxybenzotriazole (1.84 g, 11.9 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (2.73 g, 14.3 mmol) at room temperature. After being stirred for 3 h, the reaction mixture was poured into water and extracted with CH_2Cl_2. The organic layer was washed with 10% aqueous citric acid, aqueous $NaHCO_3$, and brine, then dried over $MgSO_4$ and evaporated to give **25** (4.77 g), which was used for the next reaction without further purification.

4.1.4. (2*S*,3*S*,5*S*)-1-(*tert*-Butoxycarbonyl)-5-{[(2*S*)-2-carbamoyl-1-pyrrolidinyl]carbonyl}-2-ethyl-3-pyrrolidinecarboxylic acid (26). To a stirred solution of **25** (4.77 g, 11.9 mmol), in MeOH (24 mL) was added 1 M LiOH (13 mL) at 0 °C. After being stirred at room temperature for 4 h, the reaction was quenched with 2 M HCl (18 mL). The organic solvent was removed by evaporation. The resulting residue was diluted with EtOH. Insoluble substance was removed by filtration. The filtrate was evaporated to yield **26** (4.56 g), which was used for the next reaction without further purification.

4.1.5. 3-Benzyl 1-*tert*-butyl (2*S*,3*S*,5*S*)-5-{[(2*S*)-2-carbamoyl-1-pyrrolidinyl]carbonyl}-2-ethyl-1,3-pyrrolidinedicarboxylate (27). To a stirred solution of **26** (4.56 g, 11.9 mmol) in DMF (24 mL) were added K_2CO_3 (1.86 g, 13.5 mmol) and benzylbromide (4.3 mL, 36 mmol) at room temperature. After being stirred for 15 h, the reaction mixture was poured into water and extracted with EtOAc. The organic layer was dried over $MgSO_4$ and evaporated to yield **27** (5.63 g), which was used for the next reaction without further purification.

4.1.6. 3-Benzyl 1-*tert*-butyl (2*S*,3*S*,5*S*)-5-{[(2*S*)-2-cyano-1-pyrrolidinyl]carbonyl}-2-ethyl-1,3-pyrrolidinedicarboxylate (28). To a stirred solution of **27** (5.63 g, 11.9 mmol) in THF (40 mL) were added pyridine (2.4 mL, 30 mmol) and trifluoroacetic anhydride (1.85 mL, 13 mmol) at 0 °C. After being stirred for 30 min, the reaction was quenched with water and the mixture extracted with EtOAc. The organic layer was successively washed with 1 M HCl, aqueous $NaHCO_3$, and brine, then dried over $MgSO_4$ and concentrated in vacuo.The resulting residue was purified by silica gel chromatography using EtOAc/hexane (1:1) as an eluant to yield **28** (2.34 g, 43% from **25**). TLC $R_f = 0.29$ (EtOAc/hexane, 1:1); ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.78–0.91 (m, 3H), 1.20–1.50 (m, 2H), 1.29 and 1.38 (s, 9H), 1.90–2.20 (m, 6H), 3.30–3.68 (m, 3H), 3.97–4.05 (m, 1H), 4.35–4.43 (m, 1H), 4.78–4.83 (m, 1H), 5.10–5.18 (m, 2H), 7.30–7.42 (m, 5H).

4.1.7. (2*S*,3*S*,5*S*)-1-(*tert*-Butoxycarbonyl)-5-{[(2*S*)-2-cyano-1-pyrrolidinyl]carbonyl}-2-ethyl-3-pyrrolidinecarboxylic acid (29). To a solution of **28** (1.27 g, 2.79 mmol) in EtOAc (5.5 mL) and THF (3 mL) was added 20% palladium hydroxide on carbon (127 mg). The mixture was vigorously stirred at room temperature under an atmospheric pressure of hydrogen for 20 min. The catalyst was removed by filtration and the filtrate was concentrated in vacuo to yield **29** (1.01 g, 99%). TLC $R_f = 0.20$ (EtOAc/hexane, 2:1); ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.86–0.98 (m, 3H), 1.20–1.50 (m, 2H), 1.29 and 1.38 (s, 9H), 1.85–2.45 (m, 6H), 3.09–3.20 (m, 1H), 3.40–3.65 (m, 2H), 3.95–4.05 (m, 1H), 4.35–4.42 (m, 1H), 4.78–4.83 (m, 1H).

4.1.8. *tert*-Butyl (2*S*,3*S*,5*S*)-5-{[(2*S*)-2-cyano-1-pyrrolidinyl]carbonyl}-3-(dimethylcarbamoyl)-2-ethyl-1-pyrrolidinecarboxylate (30). To a stirred solution of **29** (300 mg, 0.82 mmol) in CH_2Cl_2 (2 mL) were added dimethylamine hydrochloride (134 mg, 1.64 mmol), triethylamine (0.40 mL, 2.9 mmol), 1-hydroxybenzotriazole (125 mg, 0.82 mmol), and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (235 mg, 1.23 mmol) at room temperature. After being stirred for 4 h, the reaction mixture was poured into water and extracted with CH_2Cl_2. The organic layer was successively washed with 10% aqueous citric acid, aqueous $NaHCO_3$, and brine, then dried over $MgSO_4$ and concentrated in vacuo. The resulting residue was recrystallized from EtOAc and hexane to yield **30** (162 mg, 50%) as a white powder. TLC $R_f = 0.59$ ($CHCl_3$/MeOH, 9:1); ^{1}H NMR (300 MHz, DMSO-d_6, 100 °C) δ 0.84 (t, $J = 7.5$ Hz, 3H), 1.25–1.40 (m, 1H), 1.37 (s, 9H), 1.44–1.60 (m, 1H), 2.00–2.33 (m, 6H), 2.83 (br s, 3H), 3.04 (br s, 3H), 3.33–3.42 (m, 1H), 3.55–3.63 (m, 1H), 4.01–4.09 (m, 1H), 4.37 (t, $J = 6.9$ Hz, 1H), 4.78–4.83 (m, 1H).

4.1.9. (2*S*,3*S*,5*S*)-5-{[(2*S*)-2-Cyano-1-pyrrolidinyl]carbonyl}-2-ethyl-*N*,*N*-dimethyl-3-pyrrolidinecarboxamide 4-methylbenzenesulfonate (4b). A solution of **30** (162 mg, 0.41 mmol) and *p*-toluenesulfonic acid (86 mg, 0.45 mmol) in EtOH (2 mL) was stirred at 90 °C for 2 h. After cooling to room temperature, the resulting precipitates were collected by filtration and dried under reduced pressure to yield **4b** (188 mg, 98%) as a white powder. TLC $R_f = 0.44$ ($CHCl_3$/MeOH, 9:1); MS (APCI, pos. 20 V) *m*/*z* 293 $(M+H)^+$; IR (KBr) 3459, 2972, 1661, 1556, 1496, 1454, 1223, 1167, 1121, 1033, 1009, 682, 567 cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.91 (t, $J = 7.4$ Hz, 3H), 1.43–1.79 (m, 2H), 1.90–2.31 (m, 5H), 2.28 (s, 3H), 2.58–2.78 (m, 1H), 2.84 (s, 3H), 3.03 (s, 3H), 3.43–3.62 (m, 3H), 3.62–3.77 (m, 1H), 4.46–4.63 (m, 1H), 4.81 (dd, $J = 7.8$, 5.3 Hz, 1H), 7.10 (d, $J = 8.0$ Hz, 2H), 7.46 (d, $J = 8.0$ Hz, 2H), 7.97–8.28 (m, 1H), 9.60–9.89 (m, 1H); HRMS (FAB) calcd for $C_{15}H_{25}N_4O_2$: 293.1978. Found: 293.1976.

4.1.10. 2-*tert*-Butyl 4-methyl (2*S*,4*S*,5*S*)-5-methyl-2,4-pyrrolidinedicarboxylate (32a) and 2-*tert*-butyl 4-methyl (2*S*,4*R*,5*S*)-5-methyl-2,4-pyrrolidinedicarboxylate (32b). To a solution of **31** (6.24 g, 16.6 mmol) in AcOH (80 mL) was added 10% palladium on carbon (1.2 g). The reaction mixture was vigorously stirred at room temperature under an atmospheric pressure of hydrogen for 9 h. The catalyst was removed by filtration and the filtrate was concentrated in vacuo. The resulting residue was diluted with EtOAc. The organic layer was washed with aqueous $NaHCO_3$, dried over $MgSO_4$, and concentrated in vacuo.The resulting residue was purified by silica gel chromatography using EtOAc/hexane (3:1) as an eluant to yield **32a** (1.91 g, 47%) and **32b** (1.15 g, 28%) as a colorless oil. **32a**: TLC $R_f = 0.36$ (EtOAc/hexane, 2:1); MS (APCI, pos. 20 V) *m*/*z* 244 $(M+H)^+$; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.15 (d, $J = 6.6$ Hz, 3H), 1.48 (s, 9H), 2.14–2.37 (m, 2H), 2.90–2.99 (m, 1H), 3.32–3.43 (m, 1H), 3.66–3.70 (m, 1H), 3.67 (s, 3H). **32b**: TLC $R_f = 0.17$ (EtOAc/hexane, 1:1); MS (APCI, pos. 20 V) *m*/*z* 244 $(M+H)^+$; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.31 (d, $J = 6.3$ Hz, 3H), 1.47 (s, 9H), 2.10–2.19 (m, 1H), 2.35–2.50 (m, 2H), 3.20–3.30 (m, 1H), 3.68–3.78 (m, 1H), 3.70 (s, 3H).

4.1.11. (2*S*,4*R*,5*S*)-1-(*tert*-Butoxycarbonyl)-4-(methoxycarbonyl)-5-methyl-2-pyrrolidinecarboxylic acid (33). A solution of **32b** (1.14 g, 4.69 mmol) in trifluoroacetic

acid (9 mL) and water (1 mL) was stirred at room temperature for 3 h. The reaction mixture was evaporated. To a stirred solution of the resulting residue in THF (10 mL) and water (10 mL) were added $NaHCO_3$ to adjust to pH 9 and then di-*tert*-butyl-dicarbonate (1.54 g, 7.04 mmol) at room temperature. After being stirred for 17 h, the reaction mixture was acidified with 10% aqueous citric acid and extracted with EtOAc. The organic layer was dried over $MgSO_4$ and evaporated to yield **33** (1.45 g), which was used for the next reaction without further purification.

4.1.12. 1-*tert*-Butyl 3-methyl (2*S*,3*R*,5*S*)-5-{[(2*S*)-2-carbamoyl-1-pyrrolidinyl]carbonyl}-2-methyl-1,3-pyrrolidinedicarboxylate (34). Compound **34** was prepared as a white powder in 65% yield from **33** according to the same procedures as described for the preparation of **25** from **24**. TLC $R_f = 0.23$ (EtOAc/MeOH, 20:1); MS (APCI, pos. 20 V) *m*/*z* 384 $(M+H)^+$; ^1H NMR (300 MHz, $CDCl_3$) δ 1.35–1.50 (m, 12H), 1.70–2.20 (m, 4H), 2.30–2.50 (m, 2H), 2.80–3.05 (m, 1H), 3.50–3.90 (m, 2H), 3.72 (s, 3H), 4.07–4.48 (m, 2H), 4.54–4.73 (m, 2H), 5.30 and 5.74 (br s, 1H), 6.83 and 6.98 (s, 1H).

4.1.13. (2*S*,3*R*,5*S*)-5-{[(2*S*)-2-(Aminocarbonyl)-1-pyrrolidinyl]carbonyl}-1-(*tert*-butoxycarbonyl)-2-methyl-3-pyrrolidinecarboxylic acid (35). To a stirred solution of **34** (430 mg, 1.12 mmol) in MeOH (3 mL) was added 2 M NaOH (0.67 mL) at 0 °C. After being stirred at room temperature for 3 h, the reaction was quenched with 2 M HCl (0.67 mL). The organic solvent was removed by evaporation. The resulting residue was diluted with EtOH. Insoluble substance was removed by filtration and the filtrate was evaporated to yield **35** (412 mg), which was used for the next reaction without further purification.

4.1.14. *tert*-Butyl (2*S*,3*R*,5*S*)-5-{[(2*S*)-2-carbamoyl-1-pyrrolidinyl]carbonyl}-3-(dimethylcarbamoyl)-2-methyl-1-pyrrolidinecarboxylate (36). Compound **36** was prepared as a white powder in 90% yield from **35** according to the same procedures as described for the preparation of **30** from **29**. TLC $R_f = 0.48$ (EtOAc/MeOH/H_2O, 3:1:1); MS (APCI, pos. 20 V) *m*/*z* 397 $(M+H)^+$; ^1H NMR (300 MHz, $CDCl_3$) δ 1.35–1.50 (m, 12H), 1.70–2.20 (m, 5H), 2.25–2.60 (m, 2H), 2.95–2.97 (m, 3H), 3.01–3.05 (m, 3H), 3.50–3.90 (m, 2H), 4.00–4.17 (m, 1H), 4.57–4.80 (m, 2H), 5.30 and 5.50 (s, 1H), 6.76 and 7.04 (s, 1H).

4.1.15. *tert*-Butyl (2*S*,3*R*,5*S*)-5-{[(2*S*)-2-cyano-1-pyrrolidinyl]carbonyl}-3-[(dimethylamino)carbonyl]-2-methyl-1-pyrrolidinecarboxylate (37). Compound **37** was prepared as a white powder in 40% yield from **36** according to the same procedures as described for the preparation of **28** from **27**. TLC $R_f = 0.31$ (EtOAc/MeOH, 10:1); MS (APCI, pos. 20 V) *m*/*z* 379 $(M+H)^+$; ^1H NMR (300 MHz, DMSO-d_6, 100 °C) δ 1.31 (d, $J = 6.3$ Hz, 3H), 1.18 (s, 9H), 1.93–2.35 (m, 6H), 3.09–3.17 (m, 1H), 3.55–3.64 (m, 2H), 3.97–4.04 (m, 1H), 4.51–4.58 (m, 1H), 4.76–4.81 (m, 1H).

4.1.16. (2*S*,3*R*,5*S*)-5-{[(2*S*)-2-Cyano-1-pyrrolidinyl]carbonyl}-*N*,*N*,2-trimethyl-3-pyrrolidinecarboxamide hydrochloride (5). To a stirred solution of **37** (153 mg, 0.40 mmol) in EtOAc (1 mL) was added 4 M HCl in EtOAc (1 mL). After being stirred for 4 h, the reaction mixture was concentrated in vacuo. The resulting solid was washed with EtOAc to yield **5** (137 mg, 100%) as a white powder. TLC $R_f = 0.58$ (CH_2Cl_2/MeOH, 5:1); MS (APCI, pos. 20 V) *m*/*z* 279 $(M+H)^+$; IR (KBr) 3423, 2944, 2244, 1639, 1508, 1452, 1403, 1256, 1191, 1156, 637 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6) δ 1.32 (d, $J = 6.5$ Hz, 3 H), 1.93–2.44 (m, 6H), 2.85 (s, 3H), 2.98 (s, 3H), 3.04–3.22 (m, 1H), 3.29–3.69 (m, 2H), 3.71–3.88 (m, 1H), 4.52–4.69 (m, 1H), 4.77–4.87 (m, 1H), 8.64 (s, 1H), 10.41 (s, 1H); HRMS (FAB) calcd for $C_{14}H_{23}N_4O_2$: 279.1821. Found: 279.1819.

4.1.17. 1-*tert*-Butyl 2-ethyl (2*S*,4*R*)-4-[(benzyloxy)methyl]-5-oxo-1,2-pyrrolidinedicarboxylate (39a) and 1-*tert*-butyl 2-ethyl (2*S*,4*S*)-4-[(benzyloxy)methyl]-5-oxo-1,2-pyrrolidinedicarboxylate (39b). To a stirred solution of lithium bis(trimethylsilyl)amide in THF (22 mL,1.0 M) was added dropwise a solution of **38** (5.15 g, 20.0 mmol) in THF (20 mL) and HMPA (5 mL) at −78 °C. After being stirred for 1 h, the reaction mixture was added to a stirred solution of benzyloxymethyl chloride (5.5 mL, 40 mmol) in THF (10 mL) at −78 °C and stirred for additional 1 h. The reaction was quenched with 1 M NH_4Cl and the mixture extracted with ^{t}BuOMe. The organic layer was successively washed with aqueous $NaHCO_3$, brine, then dried over $MgSO_4$ and concentrated in vacuo. The resulting residue was purified by silica gel chromatography using EtOAc/hexane (1:2) as an eluant to yield **39a** (2.20 g, 27%) and **39b** (1.37 g, 18%) as a colorless oil. **39a**: TLC $R_f = 0.35$ (EtOAc/hexane, 1:2); MS (APCI, pos. 20 V) *m*/*z* 378 $(M+H)^+$; ^1H NMR (300 MHz, $CDCl_3$) δ 1.24 (t, $J = 7.1$ Hz, 3H), 1.49 (s, 9H), 1.99–2.13 (m, 1H), 2.45–2.60 (m, 1H), 2.80–2.94 (m, 1H), 3.66 (dd, $J = 9.3$, 7.3 Hz, 1H), 3.76 (dd, $J = 9.3$, 4.2 Hz, 1H), 4.04–4.24 (m, 2H), 4.44–4.59 (m, 3H), 7.17–7.45 (m, 5H). **39b**: TLC $R_f = 0.42$ (EtOAc/hexane, 1:2); MS (APCI, pos. 20 V) *m*/*z* 378 $(M+H)^+$; ^1H NMR (300 MHz, $CDCl_3$) δ 1.27 (q, $J = 7.1$ Hz, 3H), 1.50 (s, 9H), 2.10–2.24 (m, 1H), 2.29–2.47 (m, 1H), 2.77–2.99 (m, 1H), 3.60–3.84 (m, 2H), 4.22 (q, $J = 7.1$ Hz, 2H), 4.42–4.66 (m, 3H), 7.21–7.41 (m, 5H).

4.1.18. 1-*tert*-Butyl 2-ethyl (2*S*,4*R*)-4-[(benzyloxy)methyl]-5-methoxy-1,2-pyrrolidinedicarboxylate (40). To a stirred solution of **39a** (1.41 g, 3.74 mmol) in THF (20 mL) was added a solution of lithium triethylborohydride in THF (4.5 mL, 1.0 M) at −78 °C. After being stirred for 30 min, the reaction was quenched with aqueous $NaHCO_3$ and the mixture warmed up to 0 °C. After the addition of 30% H_2O_2 (2 mL), the reaction mixture was stirred at 0 °C. After being stirred for 30 min, the reaction mixture was evaporated to remove organic solvent and extracted with ^{t}BuOMe. The organic layer was dried over $MgSO_4$ and concentrated in vacuo. To a stirred solution of the resulting residue in MeOH (20 mL) was added *p*-toluenesulfonic acid (142 mg, 0.74 mmol) at room temperature. After being stirred for 18 h, the

reaction was quenched with aqueous $NaHCO_3$. The reaction mixture was evaporated to remove organic solvent and extracted with ^{t}BuOMe. The organic layer was washed with brine, dried over $MgSO_4$, and concentrated in vacuo to yield **40** (1.71 g), which was used for the next reaction without further purification.

4.1.19. 1-*tert*-Butyl 2-ethyl (2*S*,4*S*,5*R*)-4-[(benzyloxy)methyl]-5-methyl-1,2-pyrrolidinedicarboxylate (41). To a stirred suspension of copper(I) bromide–dimethylsulfide complex (3.58 g, 17.4 mmol) in Et_2O (34 mL) was added MeMgBr in Et_2O (5.8 mL, 3.0 M) at −40 °C. After being stirred for 1 h, the reaction mixture was cooled to −78 °C and treated with boron trifluoride-etherate (2.2 mL, 17 mmol). After being stirred for 30 min, to the above-described reaction mixture was added a solution of **40** (1.71 g, 3.74 mmol) in Et_2O (6 mL). After being stirred for 15 min, the reaction mixture was warmed up to room temperature. After 1 h, the reaction was quenched with a mixture of saturated NH_4Cl aq (10 mL) and 28% NH_3 aq (10 mL). After being stirred for 30 min, the reaction mixture was extracted with ^{t}BuOMe. The organic layer was successively washed with H_2O, brine, then dried over $MgSO_4$ and concentrated in vacuo. To a stirred solution of the resulting residue in THF (10 mL) were added aqueous $NaHCO_3$and di-*tert*-butyl-dicarbonate (816 mg, 3.74 mmol) at room temperature. After being stirred for 3 h, the reaction mixture was extracted with EtOAc. The organic layer was washed with brine, then dried over $MgSO_4$ and concentrated in vacuo. The resulting residue was purified by silica gel chromatography using EtOAc/hexane (3:7) as an eluant to yield **41** (732 mg, 45%) as a colorless oil. TLC R_f = 0.57 (EtOAc/hexane, 7:3); MS (APCI, pos. 20 V) *m*/*z* 378 $(M+H)^+$; ^{1}H NMR (300 MHz, DMSO-d_6, 100 °C) δ 1.18 (t, J = 7.0 Hz, 3H), 1.22 (d, J = 6.2 Hz, 3H), 1.37 (s, 9H), 1.67–1.78 (m, 1H), 2.06–2.20 (m, 1H), 2.37–2.47 (m, 1H), 3.37 (dd, J = 9.7, 7.3 Hz, 1H), 3.51 (dd, J = 9.7, 7.5 Hz, 1H), 3.65–3.77 (m, 1H), 4.01–4.13 (m, 2H), 4.19 (dd, J = 9.8, 4.1 Hz, 1H), 4.39–4.53 (m, 2H), 7.21–7.39 (m, 5H).

4.1.20. 1-*tert*-Butyl 2-ethyl (2*S*,4*S*,5*R*)-4-(hydroxymethyl)-5-methyl-1,2-pyrrolidinedicarboxylate (42). To a solution of **41** (732 mg, 1.94 mmol) in EtOH (10 mL) and AcOH (1 mL) was added 10% palladium on carbon (200 mg). The reaction mixture was vigorously stirred at room temperature under an atmospheric pressure of hydrogen for 3 h. The catalyst was removed by filtration and the filtrate was concentrated in vacuo to yield **42** (566 mg, 100%) as a colorless oil. TLC R_f = 0.53 (CH_2Cl_2/MeOH, 9:1); MS (APCI, pos. 20 V) *m*/*z* 378 $(M+H)^+$; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.19–1.35 (m, 6H), 1.37–1.50 (m, 9H), 1.74–1.95 (m, 1H), 1.97–2.18 (m, 1H), 2.39–2.58 (m, 1H), 3.48–3.64 (m, 1H), 3.65–3.80 (m, 1H), 3.83–4.03 (m, 1H), 4.05–4.40 (m, 3H).

4.1.21. (2*R*,3*S*,5*S*)-1-(*tert*-Butoxycarbonyl)-5-(ethoxycarbonyl)-2-methyl-3-pyrrolidinecarboxylic acid (43). To a stirred solution of **42** (566 mg, 1.94 mmol) in acetone (5 mL) was added Jones reagent (1 mL) at 0 °C. After being stirred for 2 h at room temperature, the reaction was quenched with ice-water. The reaction mixture was extracted with EtOAc. The organic layer was successively washed with H_2O, brine, then dried over $MgSO_4$ and concentrated in vacuo to yield **43** (544 mg, 93%) as a colorless oil. R_f = 0.47 (CH_2Cl_2/MeOH, 9:1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.21–1.33 (m, 6H), 1.37–1.50 (m, 9H), 2.44–2.52 (m, 2H), 2.64–2.76 (m, 1H), 4.00–4.64 (m, 4H).

4.1.22. 1-*tert*-Butyl 2-ethyl (2*S*,4*S*,5*R*)-4-(dimethylcarbamoyl)-5-methyl-1,2-pyrrolidinedicarboxylate (44). Compound **44** was prepared as a colorless oil in 70% yield from **43** according to the same procedures as described for the preparation of **30** from **29**. TLC R_f = 0.35 (acetone/hexane, 1:2); ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.09–1.28 (m, 6H), 1.28–1.42 (m, 9H), 1.83–1.97 (m, 1H), 2.36–2.46 (m, 1H), 2.80 (s, 3H), 2.98 (s, 3H), 3.00–3.12 (m, 1H), 3.83–3.97 (m, 1H), 3.96–4.14 (m, 2H), 4.21 (dd, J = 8.6, 5.9 Hz, 1H).

4.1.23. (2*S*,4*S*,5*R*)-1-(*tert*-Butoxycarbonyl)-4-(dimethylcarbamoyl)-5-methyl-2-pyrrolidinecarboxylic acid (45). To a stirred solution of **44** (200 mg, 0.61 mmol) in MeOH (2 mL) was added 1 M NaOH (1.2 mL) at room temperature. After being stirred at 60 °C for 3 h, the reaction mixture was cooled to 0 °C and the reaction was quenched with 1 M HCl (1.2 mL). The organic solvent was removed by evaporation. The resulting residue was extracted with CH_2Cl_2. The organic layer was washed with brine, dried over $MgSO_4$, and concentrated in vacuo to yield **45** (159 mg, 87%) as a colorless oil. TLC R_f = 0.46 (EtOAc/AcOH, 9:1); ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.24 (d, J = 6.0 Hz, 3H), 1.30–1.41 (m, 9H), 1.80–1.92 (m, 1H), 2.35–2.46 (m, 1H), 2.81 (s, 3H), 2.99 (s, 3H), 3.01–3.10 (m, 1H), 3.82–3.95 (m, 1H), 4.12 (t, J = 7.8 Hz, 1H), 12.33–12.54 (m, 1H).

4.1.24. *tert*-Butyl (2*R*,3*S*,5*S*)-5-{[(2*S*)-2-cyano-1-pyrrolidinyl]carbonyl}-3-(dimethylcarbamoyl)-2-methyl-1-pyrrolidinecarboxylate (46). To a stirred solution of **45** (159 g, 0.53 mmol) in CH_2Cl_2 (2 mL) were added (2*S*)-2-cyanopyrrolidine 4-methylbenzenesulfonate (116 mg, 0.61 mmol), 1-hydroxybenzotriazole (74 mg, 0.61 mmol), triethylamine (0.085 mL, 0.61 mmol), and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (116 mg, 0.61 mmol) at room temperature. After being stirred for 15 h, the reaction mixture was poured into water and extracted with CH_2Cl_2. The organic layer was successively washed with 1 M HCl, aqueous $NaHCO_3$, and brine, then dried over $MgSO_4$ and concentrated in vacuo. The resulting residue was purified by silica gel chromatography using EtOAc/hexane (3:7) as an eluant to yield **46** (130 mg, 75%) as a colorless oil. TLC R_f = 0.38 (EtOAc/MeOH, 9:1); ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.29 (d, J = 6.0 Hz, 3H), 1.35 (s, 9H), 1.55–1.80 (m, 1H), 1.98–2.25 (m, 4H), 2.40–2.56 (m, 1H), 2.91–2.97 (m, 6H), 2.96–3.07 (m, 1H), 3.45–3.67 (m, 2H), 3.90–4.12 (m, 1H), 4.49 (dd, J = 8.8, 7.7 Hz, 1H), 4.71–4.81 (m, 1H).

4.1.25. (2*R*,3*S*,5*S*)-5-{[(2*S*)-2-Cyano-1-pyrrolidinyl]carbonyl}-*N*,*N*,2-trimethyl-3-pyrrolidinecarboxamide 4-methylbenzenesulfonate (6). Compound **6** was prepared as a white powder in 57% yield from **46** according to the same procedures as described for the preparation of **4b**

from **30**. TLC $R_f = 0.35$ ($CHCl_3$/MeOH, 5:1); MS (APCI, pos. 20 V) *m/z* 279 $(M+H)^+$; IR (KBr) 3057, 2239, 1663, 1646, 1619, 1455, 1369, 1225, 1167, 682 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6) δ 1.29 (d, $J = 6.5$ Hz, 3H), 1.61–1.80 (m, 1H), 1.94–2.08 (m, 2H), 2.08–2.25 (m, 2H), 2.28 (s, 3H), 2.84 (s, 3H), 2.87–2.99 (m, 1H), 3.02 (s, 3H), 3.07–3.26 (m, 1H), 3.55 (t, $J = 6.5$ Hz, 2H), 3.73–3.85 (m, 1H), 4.49–4.66 (m, 1H), 4.82 (dd, $J = 7.8$, 4.7 Hz, 1H), 7.10 (d, $J = 8.0$ Hz, 2H), 7.47 (d, $J = 8.0$ Hz, 2H), 8.86–9.41 (m, 2H); Anal. Calcd for $C_{21}H_{30}N_4O_5S$: C, 55.98; H, 6.71; N, 12.43. Found: C, 55.83; H, 6.80; N, 12.27.

4.1.26. 1-*tert*-Butyl 2-ethyl (2*S*,4*S*)-4-[(benzyloxy)methyl]-5-methoxy-1,2-pyrrolidinedicarboxylate (47). Compound **47** was prepared from **39b** according to the same procedures as described for the preparation of **40** from **39a**, which was used for the next reaction without further purification.

4.1.27. 1-*tert*-Butyl 2-ethyl (2*S*,4*R*)-4-[(benzyloxy)methyl]-5-methyl-1,2-pyrrolidinedicarboxylate (48). Compound **48** was prepared as a colorless oil in 41% from **47** according to the same procedures as described for the preparation of **41** from **40**. TLC $R_f = 0.65$ (acetone/hexane, 1:2); ^1H NMR (300 MHz, DMSO-d_6, 100 °C) δ 0.95–1.32 (m, 6H), 1.38 (s, 9H), 1.73–2.69 (m, 3H), 3.31–3.55 (m, 2H), 3.61–4.28 (m, 4H), 4.42–4.55 (m, 2H), 7.14–7.43 (m, 5H).

4.1.28. 1-*tert*-Butyl 2-ethyl (2*S*,4*R*)-4-(hydroxymethyl)-5-methyl-1,2-pyrrolidinedicarboxylate (49). Compound **49** was prepared as a colorless oil in 83% from **48** according to the same procedures as described for the preparation of **42** from **41**. TLC $R_f = 0.42$ (acetone/hexane, 1:1); ^1H NMR (300 MHz, $CDCl_3$) δ 1.03–1.39 (m, 3H), 1.27 (q, $J = 7.1$ Hz, 3H), 1.39–1.50 (m, 9H), 1.84–2.79 (m, 3H), 3.51–3.76 (m, 2H), 4.04–4.39 (m, 4H).

4.1.29. (3*R*,5*S*)-1-(*tert*-Butoxycarbonyl)-5-(ethoxycarbonyl)-2-methyl-3-pyrrolidinecarboxylic acid (50). Compound **50** was prepared as a colorless oil in 90% from **49** according to the same procedures as described for the preparation of **43** from **42**. TLC $R_f = 0.44$ (CH_2Cl_2/MeOH, 9:1); ^1H NMR (300 MHz, $CDCl_3$) δ 1.02–1.21 (m, 3H), 1.22–1.33 (m, 3H), 1.38–1.50 (m, 9H), 2.00–2.29 (m, 1H), 2.42–2.70 (m, 1H), 3.26–3.57 (m, 1H), 4.05–4.55 (m, 4H).

4.1.30. 1-*tert*-Butyl 2-ethyl (2*S*,4*R*,5*R*)-4-(dimethylcarbamoyl)-5-methyl-1,2-pyrrolidinedicarboxylate (51). Compound **51** was prepared as a colorless oil in 70% yield from **50** according to the same procedures as described for the preparation of **30** from **29**. TLC $R_f = 0.40$ (acetone/hexane, 1:2); ^1H NMR (300 MHz, $CDCl_3$) δ 0.96–1.10 (m, 3H), 1.22–1.33 (m, 3H), 1.37–1.51 (m, 9H), 1.85–2.00 (m, 1H), 2.75–2.94 (m, 1H), 2.93–2.99 (m, 3H), 3.04–3.11 (m, 3H), 3.34–3.54 (m, 1H), 4.05–4.50 (m, 4H).

4.1.31. (2*S*,4*R*,5*R*)-1-(*tert*-Butoxycarbonyl)-4-(dimethylcarbamoyl)-5-methyl-2-pyrrolidinecarboxylic acid (52). Compound **52** was prepared as a white powder in 81% yield from **51** according to the same procedures as described for the preparation of **45** from **44**. TLC $R_f = 0.50$ (EtOAC/AcOH, 10:1); ^1H NMR (300 MHz, DMSO-d_6) δ 0.79–0.94 (m, 3H), 1.29–1.43 (m, 9H), 1.68–1.87 (m, 1H), 2.53–2.75 (m, 1H), 2.81 (s, 3H), 2.98 (s, 3H), 3.32–3.44 (m, 1H), 4.08–4.30 (m, 2H).

4.1.32. *tert*-Butyl (2*R*,3*R*,5*S*)-5-{[(2*S*)-2-cyano-1-pyrrolidinyl]carbonyl}-3-(dimethylcarbamoyl)-2-methyl-1-pyrrolidinecarboxylate (53). Compound **53** was prepared as a white powder in 75% yield from **52** according to the same procedures as described for the preparation of **46** from **45**. TLC $R_f = 0.38$ (acetone/hexane, 1:1); ^1H NMR (300 MHz, DMSO-d_6) δ 0.95 (d, $J = 6.6$ Hz, 3H), 1.35 (s, 9H), 1.72 (dd, $J = 12.7$, 6.1 Hz, 1H), 2.00–2.11 (m, 2H), 2.13–2.23 (m, 2H), 2.63–2.77 (m, 1H), 2.80–3.08 (m, 6H), 3.42–3.64 (m, 3H), 4.19–4.34 (m, 1H), 4.49 (d, $J = 9.2$ Hz, 1H), 4.70–4.81 (m, 1H).

4.1.33. (2*R*,3*R*,5*S*)-5-{[(2*S*)-2-Cyano-1-pyrrolidinyl]carbonyl}-*N*,*N*,2-trimethyl-3-pyrrolidinecarboxamide 4-methylbenzenesulfonate (7). Compound **7** was prepared as a white powder in 75% yield from **53** according to the same procedures as described for the preparation of **4b** from **30**. TLC $R_f = 0.45$ ($CHCl_3$/MeOH, 5:1); MS (APCI, pos. 20 V) *m/z* 279 $(M+H)^+$; IR (KBr) 3449, 2957, 2243, 1667, 1644, 1217, 1204, 1186, 1169, 567 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6) δ 1.19 (d, $J = 6.8$ Hz, 3H), 1.91–2.08 (m, 3H), 2.08–2.26 (m, 2H), 2.28 (s, 3H), 2.65–2.80 (m, 1H), 2.87 (s, 3H), 3.02 (s, 3H), 3.49–3.64 (m, 2H), 3.64–3.75 (m, 1H), 3.83–4.01 (m, 1H), 4.60–4.74 (m, 1H), 4.83 (dd, $J = 8.0$, 4.9 Hz, 1H), 7.10 (d, $J = 8.1$ Hz, 2H), 7.46 (d, $J = 8.1$ Hz, 2H), 8.99 (s, 1H), 9.20 (s, 1H); Anal. Calcd for $C_{21}H_{30}N_4O_5S$: C, 55.98; H, 6.71; N, 12.43. Found: C, 55.27; H, 6.71; N, 12.03.

4.1.34. *tert*-Butyl (2*S*,3*S*,5*S*)-5-{[(2*S*)-2-carbamoyl-1-pyrrolidinyl]carbonyl}-3-(diazoacetyl)-2-methyl-1-pyrrolidinecarboxylate (55). To a stirred solution of **54** (100 mg, 0.23 mmol) in THF (2 mL) were added triethylamine (0.038 mL, 0.27 mmol) and ethyl chloroformate (0.026 mL, 0.27 mmol) at 0 °C. After being stirred at room temperature for 2 h, the reaction mixture was treated with a solution of diazomethane in Et_2O and stirred for 2 h. The reaction was quenched with H_2O and the mixture extracted with CH_2Cl_2. The organic layer was dried over $MgSO_4$ and concentrated in vacuo. The resulting residue was purified by silica gel chromatography using EtOAc/MeOH (6:1) as an eluant to yield **55** (38 mg, 42%) as a colorless oil. TLC $R_f = 0.32$ (EtOAc/MeOH, 5:1); MS (APCI, pos. 20 V) *m/z* 394 $(M+H)^+$; ^1H NMR (300 MHz, $CDCl_3$) δ 1.18–1.30 (m, 3H), 1.35–1.50 (m, 9H), 1.70–2.60 (m, 6H), 3.04–3.17 (m, 1H), 3.50–3.80 (m, 2H), 4.10–4.50 (m, 2H), 4.55–4.72 (m, 1H), 5.23–5.54 (m, 2H), 6.97–7.12 (m, 1H).

4.1.35. *tert*-Butyl (2*S*,3*R*,5*S*)-5-{[(2*S*)-2-carbamoyl-1-pyrrolidinyl]carbonyl}-3-(2-methoxy-2-oxoethyl)-2-methyl-1-pyrrolidinecarboxylate (56). To a stirred solution of **55** (279 mg, 0.71 mmol) in MeOH (2 mL) were added triethylamine (0.10 mL, 0.72 mmol) and silver benzoate (8 mg, 0.03 mmol) at room temperature. After being stirred for 1 h, the reaction mixture was concentrated

in vacuo. The resulting residue was purified by silica gel chromatography using EtOAc/MeOH (6:1) as an eluant to yield **56** (219 mg, 78%) as a colorless oil. TLC $R_f = 0.48$ (EtOAc/MeOH, 5:1); MS (APCI, pos. 20 V) *m*/*z* 398 $(M+H)^+$; ^1H NMR (300 MHz, DMSO-d_6, 100 °C) δ 1.03 (d, $J = 6.6$ Hz, 3H), 1.35 (s, 9H), 1.42–1.63 (m, 1H), 1.83–2.06 (m, 5H), 2.26–2.58 (m, 3H), 3.46–3.58 (m, 2H), 3.63 (s, 3H), 3.92–4.04 (m, 1H), 4.26–4.52 (m, 2H), 6.68 (s, 2H).

4.1.36. [(2*S*,3*R*,5*S*)-1-(*tert*-Butoxycarbonyl)-5-{[(2*S*)-2-carbamoyl-1-pyrrolidinyl]carbonyl}-2-methyl-3-pyrrolidinyl]acetic acid (57). Compound **57** was prepared from **56** according to the same procedures as described for the preparation of **45** from **44**. This compound was used for the next reaction without further purification.

4.1.37. *tert*-Butyl(2*S*,3*R*,5*S*)-5-{[(2*S*)-2-carbamoyl-1-pyrrolidinyl]carbonyl}-3-[2-(dimethylamino)-2-oxoethyl]-2-methyl-1-pyrrolidinecarboxylate (58). Compound **58** was prepared as a white powder in 85% yield from **57** according to the same procedures as described for the preparation of **30** from **29**. TLC $R_f = 0.50$ (EtOAc/MeOH/AcOH, 5:1:0.1); MS (APCI, pos. 20 V) *m*/*z* 398 $(M+H)^+$; ^1H NMR (300 MHz, $CDCl_3$) δ 1.11–1.23 (m, 3H), 1.36–1.49 (m, 9H), 1.52–1.73 (m, 1H), 1.81–2.83 (m, 8H), 2.92–2.98 (m, 3H), 2.98–3.03 (m, 3H), 3.43–3.84 (m, 2H), 4.07–4.73 (m, 3H), 5.22–5.57 (m, 1H), 6.86–7.09 (m, 1H).

4.1.38. *tert*-Butyl (2*S*,3*R*,5*S*)-5-{[(2*S*)-2-cyano-1-pyrrolidinyl]carbonyl}-3-[2-(dimethylamino)-2-oxoethyl]-2-methyl-1-pyrrolidinecarboxylate (59). Compound **59** was prepared as a white powder in 40% yield from **58** according to the same procedures as described for the preparation of **28** from **27**. TLC $R_f = 0.30$ (hexane/acetone, 1:1); MS (APCI, pos. 20 V) *m*/*z* 393 $(M+H)^+$; ^1H NMR (300 MHz, DMSO-d_6, 100 °C) δ 1.03 (d, $J = 6.6$ Hz, 3H), 1.35 (s, 9H), 1.42–1.57 (m, 1H), 2.00–2.20 (m, 4H), 2.24–2.60 (m, 3H), 3.50–3.70 (m, 2H), 3.98–4.08 (m, 1H), 4.55 (dd, $J = 7.2$, 6.6 Hz, 1H), 4.70–4.80 (m, 1H).

4.1.39. 2-((2*S*,3*R*,5*S*)-5-{[(2*S*)-2-Cyano-1-pyrrolidinyl]-carbonyl}-2-methyl-3-pyrrolidinyl)-*N*,*N*-dimethylacetamide 4-methylbenzenesulfonate (8). Compound **8** was prepared as a white powder in 100% yield from **59** according to the same procedures as described for the preparation of **4b** from **30**. TLC $R_f = 0.30$ ($CHCl_3$/MeOH, 5:1); MS (APCI, pos. 20 V) *m*/*z* 293 $(M+H)^+$; IR (KBr) 2949, 2242, 1645, 1452, 1221, 1122, 1033, 1009, 682, 568 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6) δ 1.13 (d, $J = 7.0$ Hz, 3H), 1.52–1.69 (m, 1H), 1.91–2.25 (m, 6H), 2.28 (s, 3H), 2.59–2.74 (m, 2H), 2.80 (s, 3H), 2.93 (s, 3H), 3.43–3.67 (m, 2H), 3.80-3.95 (m, 1H), 4.40-4.55 (m, 1H), 4.80 (dd, $J = 7.9$, 4.9 Hz, 1H), 7.10 (d, $J = 8.1$ Hz, 2H), 7.46 (d, $J = 8.1$ Hz, 2H), 8.25 (s, 1H), 9.58 (s, 1H).

4.1.40. 4-Benzyl 1-*tert*-butyl 2-methyl (2*S*)-5-methyl-2,3-dihydro-1*H*-pyrrole-1,2,4-tricarboxylate (61). Compound **61** was prepared as a white powder in 82% yield from **60** according to the same procedures as described for the preparation of **22** from **21**. TLC $R_f = 0.55$ (EtOAc/hexane, 1:3); ^1H NMR (300 MHz, $CDCl_3$) δ 1.45 (s, 9H), 2.64 (s, 3H), 2.69–2.80 (m, 1H), 2.97–3.30 (m, 1H), 3.75 (s, 3H), 4.67 (dd, $J = 12.4$, 5.2 Hz, 1H), 5.16 (s, 2H), 7.16–7.48 (m, 5H).

4.1.41. (5*S*)-1-(*tert*-Butoxycarbonyl)-5-(methoxycarbonyl)-2-methyl-4,5-dihydro-1*H*-pyrrole-3-carboxylic acid (62). To a solution of **61** (230 g, 613 mmol) in MeOH (900 mL) was added 10% palladium on carbon (23 g). The mixture was vigorously stirred at room temperature under an atmospheric pressure of hydrogen. The catalyst was removed by filtration and the filtrate was concentrated in vacuo. The resulting residue was recrystallized from $^iPr_2O$–hexane to yield **62** (161 g, 92%) as a white powder. TLC $R_f = 0.25$ (EtOAc/hexane, 1:3); ^1H NMR (300 MHz, $CDCl_3$) δ 1.46 (s, 9H), 2.64 (s, 3H), 2.67–2.77 (m, 1H), 2.93–3.19 (m, 1H), 3.77 (s, 3H), 4.69 (dd, $J = 12.5$, 5.1 Hz, 1H).

4.1.42. 1-*tert*-Butyl 2-methyl (2*S*)-4-(dimethylcarbamoyl)-5-methyl-2,3-dihydro-1*H*-pyrrole-1,2-dicarboxylate (63). Compound **63** was prepared as a white powder in 100% yield from **62** according to the same procedures as described for the preparation of **30** from **29** TLC $R_f = 0.57$ (CH_2Cl_2/MeOH, 10:1); ^1H NMR (300 MHz, $CDCl_3$) δ 1.45 (s, 9H), 2.17 (s, 3H), 2.57-2.69 (m, 1H), 2.99 (s, 6H), 3.09–3.29 (m, 1H), 3.76 (s, 3H), 4.69 (dd, $J = 12.0$, 4.9 Hz, 1H).

4.1.43. 1-*tert*-Butyl 2-methyl (2*S*,4*S*,5*S*)-4-(dimethylcarbamoyl)-5-methyl-1,2-pyrrolidinedicarboxylate (64). To a solution of **63** (11 g, 35.1 mmol) in AcOH (120 mL) was added platinum(IV) oxide (3.0 g, 13.2 mmol). The mixture was vigorously stirred at room temperature under an atmospheric pressure of hydrogen for 5 h. The catalyst was removed by filtration and the filtrate was concentrated in vacuo. The resulting residue was dissolved in EtOAc. The solution was successively washed with water, aqueous $NaHCO_3$, and brine, then dried over $MgSO_4$ and concentrated in vacuo to yield **64** (10.3 g, 93%) as a colorless oil. TLC $R_f = 0.55$ (CH_2Cl_2/MeOH, 10:1); ^1H NMR (300 MHz, $CDCl_3$) δ 1.07–1.19 (m, 3 H), 1.38–1.52 (m, 9H), 2.21–2.37 (m, 1H), 2.53–2.77 (m, 1H), 2.93–3.01 (m, 3H), 3.03–3.13 (m, 3H), 3.17–3.38 (m, 1H), 3.69–3.80 (m, 3H), 4.06–4.45 (m, 2H).

4.1.44. (2*S*,4*S*,5*S*)-1-(*tert*-Butoxycarbonyl)-4-(dimethylcarbamoyl)-5-methyl-2-pyrrolidinecarboxylic acid (65). To a stirred solution of **64** (10.3 g, 32.8 mmol) in THF (100 mL) was added 2 M NaOH (21.3 mL) at room temperature. After being stirred for 23 h, the reaction was quenched with 2 M HCl (21.3 mL). The reaction mixture was extracted with CH_2Cl_2. The organic layer was washed with brine, dried over $MgSO_4$, concentrated in vacuo, and recrystallized from EtOAc to yield **65** (5.62 g, 57%) as a white powder. TLC $R_f = 0.36$ (CH_2Cl_2/MeOH, 10:1); ^1H NMR (300 MHz, $CDCl_3$) δ 1.12 (d, $J = 6.6$ Hz, 3H), 1.44 (s, 9H), 2.25-2.44 (m, 1H), 2.58–2.83 (m, 1H), 2.98 (s, 3H), 3.08 (s, 3H), 3.17–3.43 (m, 1H), 4.12–4.56 (m, 2H), 8.11 (s, 1H).

4.1.45. (2*S*,4*S*,5*S*)-1-[(Benzyloxy)carbonyl]-4-(dimethylcarbamoyl)-5-methyl-2-pyrrolidinecarboxylic acid (66). To a stirred solution of **65** (5.62 g, 18.7 mmol) in CH_2Cl_2 (20 mL) was added 4 N HCl in dioxane (45 mL) at room temperature. After being stirred for 1 h, the reaction mixture was concentrated in vacuo. To a stirred solution of the resulting residue in H_2O (20 mL) $^iPr_2O$ (5 mL) were added $NaHCO_3$ (6.28 g, 74.8 mmol) and a solution of benzyloxycarbonyl chloride (3.0 mL, 21 mmol) in $^iPr_2O$ (15 mL). After being stirred for 15 h, the reaction was quenched with 2 M HCl and the mixture extracted with CH_2Cl_2. The organic layer was washed with brine, dried over MgSO4, and concentrated in vacuo to yield **66** (5.44 g, 87%) as a white powder. TLC $R_f = 0.22$ (CH_2Cl_2/MeOH, 10:1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.15 (d, $J = 6.2$ Hz, 3H), 2.32–2.51 (m, 1H), 2.64–2.81 (m, 1H), 2.98 (s, 3H), 3.07 (s, 3H), 3.21–3.36 (m, 1H), 4.20–4.55 (m, 2H), 5.02–5.26 (m, 2H), 7.20–7.44 (m, 5H).

4.1.46. Benzyl (2*S*,3*S*,5*S*)-5-[(2-amino-2-oxoethyl)(methyl)carbamoyl]-3-(dimethylcarbamoyl)-2-methyl-1-pyrrolidinecarboxylate (67). To a stirred solution of **66** (340 mg, 1.02 mmol) in CH_2Cl_2 (7 mL) were added oxalyl chloride (0.10 mL, 1.12 mmol) and DMF (one drop) at room temperature. After being stirred for 20 min, the reaction mixture was concentrated in vacuo. To a stirred solution of the resulting residue in CH_2Cl_2 (5 mL) were added *N*-methylglycinamide hydrochloride (152 mg, 1.22 mmol) and triethylamine (0.36 mmol, 2.54 mmol). After being stirred for 1 h, the reaction mixture was poured into water and extracted with CH_2Cl_2. The organic layer was washed with 10% aqueous citric acid, aqueous $NaHCO_3$, and brine, dried over $MgSO_4$, and evaporated to give **67** (270 mg, 66%). TLC $R_f = 0.36$ (EtOAc/MeOH, 10:1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.01–1.48 (m, 6H), 2.09–2.39 (m, 1H), 2.53–2.80 (m, 1H), 2.78–3.13 (m, 9H), 3.24–3.40 (m, 1H), 4.19–5.51 (m, 5H), 6.20 and 6.40 (s, 1H), 7.24–7.41 (m, 5H), 7.68 (s, 1H).

4.1.47. *tert*-Butyl (2*S*,3*S*,5*S*)-5-[(cyanomethyl)carbamoyl]-3-(dimethylcarbamoyl)-2-methyl-1-pyrrolidinecarboxylate (69a). To a stirred solution of **65** (300 mg, 1.0 mmol) in CH_2Cl_2 (4 mL) were added pyridine (0.25 mL, 3.0 mmol) and cyanuric fluoride (0.085 mL, 1.0 mmol) at 0 °C. After being stirred for 1 h, the reaction was quenched with ice-water. Insoluble substance was removed by filtration. The organic layer was dried over $MgSO_4$ and concentrated in vacuo. To a stirred solution of the resulting residue in 1,2-dichloroethane (4 mL) were added aminoacetonitrile (84 mg, 1.5 mmol) and pyridine (0.16 mL, 2 mmol) at room temperature. After being stirred for 30 min at 60 °C, the reaction was quenched with 10% aqueous citric acid and the mixture extracted with CH_2Cl_2. The organic layer was successively washed with aqueous $NaHCO_3$, brine, then dried over Na_2SO_4 and concentrated in vacuo. The resulting solid was washed with ^{t}BuOMe to yield **69a** (250 mg, 74%) as a white powder. TLC $R_f = 0.41$ (CH_2Cl_2/MeOH, 9:1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.14 (d, $J = 6.6$ Hz, 3H), 1.47 (s, 9H), 2.25–2.42 (m, 1H), 2.54–2.79 (m, 1H), 2.97 (s, 3H), 3.08 (s, 3H), 3.22–3.34 (m, 1H), 3.97–4.12 (m, 1H), 4.17 (dd, $J = 10.4$, 7.7 Hz, 1H), 4.26–4.47 (m, 2H), 6.38–6.82 (m, 1H).

4.1.48. *tert*-Butyl (2*S*,3*S*,5*S*)-5-[(cyanomethyl)(methyl)carbamoyl]-3-(dimethylcarbamoyl)-2-methyl-1-pyrrolidinecarboxylate (69b). To a solution of **67** (261 mg, 0.645 mmol) in THF (6 mL) was added 10% palladium on carbon (55 mg). After being stirred at room temperature under an atmospheric pressure of hydrogen for 2 h, the catalyst was removed by filtration. To the filtrate was added a solution of di-*tert*-butyl-dicarbonate (169 mg, 0.774 mmol) in THF (6 mL). After being stirred for 1 h, the reaction mixture was treated with pyridine (0.26 mL, 3.23 mmol) and trifluoroacetic anhydride (0.14 mL, 0.97 mmol) and stirred for additional 30 min. The reaction was quenched with water and the mixture extracted with EtOAc. The organic layer was successively washed with 1 M HCl, aqueous $NaHCO_3$, and brine, then dried over $MgSO_4$ and concentrated in vacuo.The resulting residue was purified by silica gel chromatography using EtOAc/MeOH (40:1) as an eluant to yield **69b** (170 mg, 75%) as a white powder. TLC $R_f = 0.31$ (CH_2Cl_2/MeOH, 10:1); ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.91 (d, $J = 6.6$ Hz, 3H), 1.26–1.39 (m, 9H), 2.11–2.23 (m, 2H), 2.81 (s, 3H), 3.01 (s, 3H), 3.11 (s, 3H), 4.06–4.31 (m, 2H), 4.36–4.67 (m, 3H).

According to the same procedures as described for the preparation of **69a** from **65**, compounds **69c**–**g** were prepared from **65**.

4.1.49. *tert*-Butyl (2*S*,3*S*,5*S*)-5-[(cyanomethyl)(ethyl)carbamoyl]-3-(dimethylcarbamoyl)-2-methyl-1-pyrrolidinecarboxylate (69c). Yield 75%. A white powder. $R_f = 0.21$ (EtOAc); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.15 (d, $J = 6.6$ Hz, 3H), 1.34 (t, $J = 7.1$ Hz, 3H), 1.39–1.49 (m, 9H), 2.17–2.37 (m, 1H), 2.53–2.71 (m, 1H), 2.96 (s, 3H), 3.05–3.13 (m, 3H), 3.23–3.39 (m, 1H), 3.47–3.71 (m, 2H), 3.98–4.70 (m, 4H).

4.1.50. *tert*-Butyl (2*S*,3*S*,5*S*)-5-[(cyanomethyl)(propyl)carbamoyl]-3-(dimethylcarbamoyl)-2-methyl-1-pyrrolidinecarboxylate (69d). Yield 82%. A white powder. $R_f = 0.28$ (EtOAc); ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.91–1.05 (m, 3H), 1.16 (d, $J = 6.6$ Hz, 3H), 1.40–1.48 (m, 9H), 1.63–1.83 (m, 2H), 2.13–2.37 (m, 1H), 2.56–2.77 (m, 1H), 2.96–2.99 (m, 3H), 3.05–3.11 (m, 3H), 3.19–3.66 (m, 3H), 3.91–4.73 (m, 4H).

4.1.51. *tert*-Butyl (2*S*,3*S*,5*S*)-5-[allyl(cyanomethyl)carbamoyl]-3-(dimethylcarbamoyl)-2-methyl-1-pyrrolidinecarboxylate (69e). Yield 80%. A white powder. $R_f = 0.29$ (EtOAc); MS (APCI, pos.) *m*/*z* 379 $(M+H)^+$; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.16 (d, $J = 6.6$ Hz, 3H), 1.40–1.51 (m, 9H), 2.15–2.29 (m, 1H), 2.56–2.77 (m, 1H), 2.96 (s, 3H), 3.03–3.13 (m, 3H), 3.18–3.35 (m, 1H), 3.90–4.78 (m, 6H), 5.24–5.44 (m, 2H), 5.70–6.07 (m, 1H).

4.1.52. *tert*-Butyl (2*S*,3*S*,5*S*)-5-[(cyanomethyl)(cyclopropyl)carbamoyl]-3-(dimethylcarbamoyl)-2-methyl-1-pyrrolidinecarboxylate (69f). Yield 73%. A white powder. $R_f = 0.24$ (EtOAc); ^{1}H NMR (300 MHz, $CDCl_3$) δ

0.81–1.10 (m, 4H), 1.12–1.22 (m, 3H), 1.41–1.48 (m, 9H), 2.20–2.41 (m, 1H), 2.50–2.68 (m, 1H), 2.80–3.02 (m, 4H), 3.05–3.14 (m, 3H), 3.23–3.40 (m, 1H), 3.99–4.16 (m, 1H), 4.17–4.46 (m, 1H), 4.49–4.62 (m, 1H), 4.88–5.05 (m, 1H).

4.1.53. *tert*-Butyl (2*S*,3*S*,5*S*)-5-[(cyanomethyl)(2-propyn-1-yl)carbamoyl]-3-(dimethylcarbamoyl)-2-methyl-1-pyrrolidinecarboxylate (69g). Yield 61%. A white powder. R_f = 0.32 (EtOAc); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.15 (d, J = 6.6 Hz, 3H), 1.34–1.51 (m, 9H), 2.25–2.51 (m, 2H), 2.55–2.76 (m, 1H), 2.97 (s, 3H), 3.05–3.14 (m, 3H), 3.19–3.42 (m, 1H), 4.06–4.83 (m, 6H).

According to the same procedures as described for the preparation of **4b** from **30**, compounds **9**–**15** were prepared from **69a**–**69g**, respectively.

4.1.54. (2*S*,4*S*,5*S*)-*N*-(Cyanomethyl)-4-(dimethylaminocarbonyl)-5-methyl-2-pyrrolidinecarboxamide 4-methylbenzenesulfonate (9). Yield 100%. A white powder. TLC R_f = 0.33 (CH_2Cl_2/MeOH, 4:1); MS (APCI, pos. 20 V) m/z 239 $(M+H)^+$; IR (KBr) 1632, 1551, 1495, 1214, 1160, 1120, 1031, 1007, 680, 566 cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.11 (d, J = 6.8 Hz, 3H), 2.13–2.27 (m, 1H), 2.28 (s, 3H), 2.39–2.48 (m, 1H), 2.83 (s, 3H), 2.99 (s, 3H), 3.57–3.68 (m, 1H), 3.86–4.00 (m, 1H), 4.18–4.35 (m, 3H), 7.11 (d, J = 7.9 Hz, 2H), 7.47 (d, J = 8.2 Hz, 2H), 8.23–8.39 (m, 1H), 9.16 (t, J = 5.6 Hz, 1H), 9.59 (s, 1H); HRMS (FAB) calcd for $C_{11}H_{19}N_4O_2$: 239.1508. Found: 239.1515.

4.1.55. (2*S*,4*S*,5*S*)-*N*-(Cyanomethyl)-*N*,5-dimethyl-4-(dimethylcarbamoyl)-2-pyrrolidinecarboxamide 4-methylbenzenesulfonate (10). Yield 100%. A white powder. TLC R_f = 0.50 (CH_2Cl_2/MeOH, 5:1); MS (APCI, pos. 20 V) m/z 253 $(M+H)^+$; IR (KBr) 3426, 2940, 2250, 1665, 1635, 1183, 1124, 1034, 1010, 685 cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.15 (d, J = 6.8 Hz, 3H), 2.02–2.17 (m, 1H), 2.28 (s, 3H), 2.56–2.71 (m, 1H), 2.83 (s, 3H), 3.00 (s, 3H), 3.06 (s, 3H), 3.56–3.68 (m, 1H), 3.81–3.95 (m, 1H), 4.39 (d, J = 18.0 Hz, 1H), 4.56 (d, J = 18.0 Hz, 1H), 4.63–4.76 (m, 1H), 7.11 (d, J = 8.1 Hz, 2H), 7.47 (d, J = 8.1 Hz, 2H), 8.04–8.22 (m, 1H), 9.57–9.71 (m, 1H).

4.1.56. (2*S*,4*S*,5*S*)-*N*-(Cyanomethyl)-*N*-ethyl-4-(dimethylcarbamoyl)-5-methyl-2-pyrrolidinecarboxamide 4-methylbenzenesulfonate (11). Yield 100%. A white powder. TLC R_f = 0.29 (CH_2Cl_2/MeOH, 4:1); MS (APCI, pos. 20 V) m/z 267 $(M+H)^+$; IR (KBr) 2975, 2939, 2245, 1654, 1637, 1217, 1162, 1120, 1008, 566 cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.05–1.27 (m, 6H), 1.99–2.19 (m, 1H), 2.28 (s, 3H), 2.52–2.70 (m, 1H), 2.83 (s, 3H), 3.00 (s, 3H), 3.32–3.52 (m, 2H), 3.63 (q, J = 7.4 Hz, 1H), 3.82–4.01 (m, 1H), 4.31–4.58 (m, 2H), 4.57–4.84 (m, 1H), 7.11 (d, J = 8.1 Hz, 2H), 7.47 (d, J = 8.1 Hz, 2H), 7.99–8.38 (m, 1H), 9.44–9.84 (m, 1H); HRMS (FAB) calcd for $C_{13}H_{23}N_4O_2$: 267.1821. Found: 267.1818.

4.1.57. (2*S*,4*S*,5*S*)-*N*-(Cyanomethyl)-4-(dimethylcarbamoyl)-5-methyl-*N*-propyl-2-pyrrolidinecarboxamide 4-methylbenzenesulfonate (12). Yield 89%. A white powder. TLC R_f = 0.53 ($CHCl_3$/MeOH, 4:1); MS (APCI, pos. 20 V) m/z 281 $(M+H)^+$; IR (KBr) 3464, 3112, 2246, 1655, 1458, 1223, 1163, 1120, 1008, 680 cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.83–0.93 (m, 3H), 1.16 (d, J = 6.8 Hz, 3H), 1.46–1.77 (m, 2H), 2.01–2.17 (m, 1H), 2.28 (s, 3H), 2.55–2.72 (m, 1H), 2.83 (s, 3H), 3.00 (s, 3H), 3.32 (t, J = 7.5 Hz, 2H), 3.59–3.72 (m, 1H), 3.85–3.97 (m, 1H), 4.31–4.63 (m, 2H), 4.59–4.78 (m, 1H), 7.11 (d, J = 8.1 Hz, 2H), 7.48 (d, J = 8.1 Hz, 2H), 8.08–8.23 (m, 1H), 9.57–9.74 (m, 1H); HRMS (FAB) calcd for $C_{14}H_{25}N_4O_2$: 281.1978. Found: 281.1988.

4.1.58. (2*S*,4*S*,5*S*)-*N*-Allyl-*N*-(Cyanomethyl)-4-(dimethylcarbamoyl)-5-methyl-2-pyrrolidinecarboxamide 4-methylbenzenesulfonate (13). Yield 90%. A white powder. TLC R_f = 0.57 ($CHCl_3$/MeOH, 4:1); MS (APCI, pos. 20 V) m/z 279 $(M+H)^+$; IR (KBr) 3448, 2246, 1661, 1636, 1560, 1496, 1224, 1120, 1008, 681 cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.16 (d, J = 6.8 Hz, 3 H), 1.99–2.19 (m, 1H), 2.28 (s, 3H), 2.53–2.69 (m, 1H), 2.83 (s, 3H), 3.00 (s, 3H), 3.56–3.70 (m, 1H), 3.84–3.95 (m, 1H), 3.96–4.17 (m, 2H), 4.21–4.53 (m, 2H), 4.59–4.76 (m, 1H), 5.22–5.39 (m, 2H), 5.73–5.96 (m, 1H), 7.11 (d, J = 8.1 Hz, 2H), 7.47 (d, J = 8.1 Hz, 2H), 8.12–8.26 (m, 1H), 9.58–9.76 (m, 1H); HRMS (FAB) calcd for $C_{14}H_{23}N_4O_2$: 279.1821. Found: 279.1819.

4.1.59. (2*S*,4*S*,5*S*)-*N*-(Cyanomethyl)-*N*-cyclopropyl-4-(dimethylcarbamoyl)-5-methyl-2-pyrrolidinecarboxamide 4-methylbenzenesulfonate (14). Yield 100%. A white powder. TLC R_f = 0.42 (CH_2Cl_2/MeOH, 9:1); MS (APCI, pos. 20 V) m/z 279 $(M+H)^+$; IR (KBr) 1639, 1442, 1364, 1226, 1161, 1119, 1031, 1008, 680, 565 cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.83–1.06 (m, 4H), 1.15 (d, J = 6.8 Hz, 3H), 2.18–2.34 (m, 1H), 2.28 (s, 3H), 2.58–2.73 (m, 1H), 2.81–2.94 (m, 1H), 2.84 (s, 3H), 3.01 (s, 3H), 3.59–3.74 (m, 1H), 3.90–4.05 (m, 1H), 4.35–4.54 (m, 2H), 4.72–4.84 (m, 1H), 7.11 (d, J = 7.9 Hz, 2H), 7.47 (d, J = 8.1 Hz, 2H), 8.07–8.31 (m, 1H), 9.33–9.64 (m, 1H); HRMS (FAB) calcd for $C_{14}H_{23}N_4O_2$: 279.1821. Found: 279.1820.

4.1.60. (2*S*,4*S*,5*S*)-*N*-(Cyanomethyl)-4-(dimethylcarbamoyl)-5-methyl-*N*-propargyl-2-pyrrolidinecarboxamide 4-methylbenzenesulfonate (15). Yield 100%. A white powder. TLC R_f = 0.44 (CH_2Cl_2/MeOH, 9:1); MS (APCI, pos. 20 V) m/z 277 $(M+H)^+$; IR (KBr) 2120, 1667, 1634, 1466, 1161, 1120, 1032, 1008, 680, 566 cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.15 (d, J = 6.8 Hz, 3H), 2.04–2.26 (m, 1H), 2.28 (s, 3H), 2.57–2.77 (m, 1H), 2.84 (s, 3H), 3.01 (s, 3H), 3.36–3.71 (m, 2H), 3.83–3.97 (m, 1H), 4.21–4.84 (m, 5H), 7.11 (d, J = 8.4 Hz, 2H), 7.47 (d, J = 8.2 Hz, 2H), 8.10–8.35 (m, 1H), 9.58–9.79 (m, 1H); HRMS (FAB) calcd for $C_{14}H_{21}N_4O_2$: 277.1665. Found: 277.1663.

4.1.61. 2-Benzyl 1-*tert*-butyl 4-methyl (2*S*)-5-methyl-2,3-dihydro-1*H*-pyrrole-1,2,4-tricarboxylate (71). Compound **71** was prepared as a yellow oil in 89% yield from **70** according to the same procedures as described for the preparation of **22** from **21**. TLC R_f = 0.69 (EtOAc/hexane,

1:2); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.36–1.45 (m, 9H), 2.63 (t, J = 1.7 Hz, 3H), 2.66–2.77 (m, 1H), 3.01–3.15 (m, 1H), 3.69 (s, 3H), 4.65–4.76 (m, 1H), 5.12–5.26 (m, 2H), 7.29–7.41 (m, 5H).

4.1.62. (2*S*)-1-(*tert*-Butoxycarbonyl)-4-(methoxycarbonyl)-5-methyl-2,3-dihydro-1*H*-pyrrole-2-carboxylic acid (72). To a solution of **71** (89.4 g, 265 mmol) in MeOH (330 mL) was added 10% palladium on carbon (8.9 g). The reaction mixture was vigorously stirred at room temperature under an atmospheric pressure of hydrogen for 2.5 h. The catalyst was removed by filtration and the filtrate was concentrated in vacuo. The resulting residue was treated with ^{i}PrOH–hexane to yield **72** (49.7 g, 66%) as a white powder. TLC R_f = 0.41 ($CHCl_3$/MeOH, 9:1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.40–1.55 (m, 9H), 1.69 (s, 1H), 2.61 (s, 3H), 2.78–2.92 (m, 1H), 3.02–3.18 (m, 1H), 3.71 (s, 3H), 4.71 (dd, J = 12.2, 4.9 Hz, 1H).

4.1.63. 1-*tert*-Butyl 3-methyl (5*S*)-5-[(2-amino-2-oxoethyl)(ethyl)carbamoyl]-2-methyl-4,5-dihydro-1*H*-pyrrole-1,3-dicarboxylate (73). To a stirred solution of **72** (4.40 g, 15.4 mmol) in CH_2Cl_2 (60 mL) were added pyridine (4.5 mL, 55 mmol) and cyanuric fluoride (1.6 mL, 19 mmol) at −10 °C. After being stirred for 1.5 h, the reaction was quenched with ice-water. Insoluble substance was removed by filtration. The organic layer was dried over $MgSO_4$ and concentrated in vacuo. To a stirred solution of the resulting residue in 1,2-dichloroethane (20 mL) were added *N*-ethylglycinamide (2.04 g, 20 mmol) and pyridine (1.6 mL, 20 mmol) at room temperature. After being stirred at 60 °C for 1.5 h, the reaction was quenched with H_2O and the mixture extracted with CH_2Cl_2. The organic layer was successively washed with 1 M HCl, aqueous $NaHCO_3$, and brine, then dried over Na_2SO_4 and concentrated in vacuo to yield **73** (5.13 g, 90%) as a pale yellow powder. TLC R_f = 0.35 (EtOAc); MS (APCI, pos. 20 V) m/z 370 $(M+H)^+$; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.30 (t, J = 7.2 Hz, 3H), 1.39–1.54 (m, 9H), 2.50–2.75 (m, 4H), 2.93–3.14 (m, 1H), 3.18–3.51 (m, 1H), 3.53–3.85 (m, 5H), 4.19–4.71 (m, 1H), 4.97 (dd, J = 11.9, 5.9 Hz, 1H), 5.26–5.48 (m, 1H), 6.54–6.83 (m, 1H).

4.1.64. 1-*tert*-Butyl 3-methyl (2*S*,3 *S*,5*S*)-5-[(2-amino-2-oxoethyl)(ethyl)carbamoyl]-2-methyl-1,3-pyrrolidinedicarboxylate (74). To a solution of **73** (5.13 g, 13.9 mmol) in AcOH (45 mL) was added platinum(IV) oxide (1.0 g, 4.4 mmol). The mixture was vigorously stirred at room temperature under an atmospheric pressure of hydrogen for 2 h. The catalyst was removed by filtration and the filtrate was concentrated in vacuo. The resulting residue was dissolved in EtOAc. The solution was successively washed with water, aqueous $NaHCO_3$, and brine, then dried over $MgSO_4$ and concentrated in vacuo to yield **74** (4.55 g, 88%) as a white powder. TLC R_f = 0.50 (CH_2Cl_2/MeOH, 10:1); MS (APCI, pos. 20 V) m/z 372 $(M+H)^+$; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.20–1.29 (m, 6H), 1.42 (s, 9H), 2.17–2.29 (m, 1H), 2.41–2.59 (m, 1H), 3.13–3.35 (m, 2H), 3.39 (d, J = 17.2 Hz, 1H), 3.73 (s, 3H), 3.79–3.93 (m, 1H), 4.19–4.31 (m, 1H), 4.57 (dd, J = 10.1, 7.1 Hz, 1H), 4.77 (d, J = 17.6 Hz, 1H), 5.24–5.37 (m, 1H), 7.24–7.35 (m, 1H).

4.1.65. (2*S*,3*S*,5*S*)-5-[(2-Amino-2-oxoethyl)(ethyl)carbamoyl]-1-(*tert*-butoxycarbonyl)-2-methyl-3-pyrrolidinecarboxylic acid (75). To a stirred solution of **74** (4.55 g, 12.2 mmol) in MeOH (25 mL) was added 1 M LiOH (18.3 mL) at 0 °C. After being stirred for 1.5 h, the reaction was quenched with 2 M HCl (9.2 mL). The organic solvent was removed by evaporation. The resulting residue was diluted with CH_2Cl_2. Insoluble substance was removed by filtration. The filtrate was evaporated to yield **75** (4.37 g), which was used for the next reaction without further purification. TLC R_f = 0.14 (CH_2Cl_2/MeOH, 10:1).

4.1.66. 3-Benzyl 1-*tert*-butyl (2*S*,3*S*,5*S*)-5-[(2-amino-2-oxoethyl)(ethyl)carbamoyl]-2-methyl-1,3-pyrrolidinedicarboxylate (76). To a stirred solution of **75** (4.37 g, 12.2 mmol) in DMF (20 mL) were added K_2CO_3 (1.94 g, 14 mmol) and benzylbromide (1.6 mL, 13.4 mmol) at room temperature. After being stirred for 4 h, the reaction mixture was poured into water and extracted with CH_2Cl_2. The organic layer was washed with brine, then dried over $MgSO_4$ and evaporated to yield **76** (5.45 g), which was used for the next reaction without further purification. TLC R_f = 0.49 (EtOAc); MS (APCI, pos. 20 V) m/z 448 $(M+H)^+$; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.11–1.31 (m, 6H), 1.36–1.51 (m, 9H), 2.18–2.38 (m, 1H), 2.43–2.61 (m, 1H), 3.15–3.33 (m, 2H), 3.38 (d, J = 16.8 Hz, 1H), 3.75–3.94 (m, 1H), 4.18–4.33 (m, 1H), 4.51–4.62 (m, 1H), 4.76 (d, J = 16.8 Hz, 1H), 5.07–5.24 (m, 2H), 5.24–5.55 (m, 1H), 7.28–7.46 (m, 6H).

4.1.67. 3-Benzyl 1-*tert*-butyl (2*S*,3*S*,5*S*)-5-[(cyanomethyl)(ethyl)carbamoyl]-2-methyl-1,3-pyrrolidinedicarboxylate (77). To a stirred solution of **76** (5.45 g, 12.2 mmol) in THF (40 mL) were added pyridine (4.9 mL, 61 mmol) and trifluoroacetic anhydride (2.6 mL, 18 mmol) at 0 °C. After being stirred at room temperature for 45 min, the reaction was quenched with water and the mixture extracted with EtOAc. The organic layer was successively washed with 1 M HCl, aqueous $NaHCO_3$, and brine, then dried over $MgSO_4$ and concentrated in vacuo.The resulting residue was purified by silica gel chromatography using EtOAc/hexane (1:1) as an eluant to yield **77** (3.42 g, 65% from **74**) as a pale yellow oil. TLC R_f = 0.38 (EtOAc/hexane, 1:1); MS (APCI, pos. 20 V) m/z 430 $(M+H)^+$; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.17 (d, J = 6.6 Hz, 3H), 1.30–1.50 (m, 12H), 2.25–2.46 (m, 2H), 3.13–3.29 (m, 1H), 3.41–3.77 (m, 2H), 3.89–4.19 (m, 1H), 4.23–4.45 (m, 1H), 4.46–4.80 (m, 2H), 5.06–5.26 (m, 2H), 7.29–7.43 (m, 5H).

4.1.68. (2*S*,3*S*,5*S*)-1-(*tert*-Butoxycarbonyl)-5-[(cyanomethyl)(ethyl)carbamoyl]-2-methyl-3-pyrrolidinecarboxylic acid (78). To a solution of **77** (3.42 g, 7.96 mmol) in EtOAc (80 mL) was added 20% palladium hydroxide on carbon (700 mg). The mixture was vigorously stirred at room temperature under an atmospheric pressure of hydrogen for 30 min. The catalyst was removed by filtration and the filtrate was concentrated in vacuo to yield **78** (2.52 g, 93%). TLC R_f = 0.28 (EtOAc/hexane, 1:1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.15–1.55 (m, 15H), 2.24–2.49 (m, 2H), 3.13–3.34 (m, 1H), 3.42–3.81 (m, 2H), 3.88–4.87 (m, 4H).

4.1.69. *tert*-Butyl (2*S*,3*S*,5*S*)-5-[(cyanomethyl)(ethyl)carbamoyl]-2-methyl-3-(1-pyrrolidinylcarbonyl)-1-pyrrolidinecarboxylate (79a). To a stirred solution of **78** (100 mg, 0.30 mmol) and 1-hydroxybenzotriazole (50 mg, 0.32 mmol) in CH_2Cl_2 (3 mL) was added polystyrene carbodiimide (PS-carbodiimide) (315 mg, 0.42). After being stirred for 1.5 h, the resin was removed by filtration. The filtrate was treated with pyrrolidine (25 mg, 0.35 mmol) and PS-NMM (185 mg, 0.32 mmol) at room temperature. After being stirred for 15 min, the reaction mixture was treated with macroporous triethylammonium methylpolystyrene carbonate (MP-carbonate) (340 mg, 0.97 mmol). After being stirred for 30 min, the reaction mixture was treated with PS-isocyanate (200 mg, 0.30 mmol). After being stirred for 2 h, the resulting insoluble substance was removed by filtration. The filtrate was concentrated in vacuo. The resulting residue was purified by silica gel chromatography using EtOAc/hexane (2:1) as an eluant to yield **79a** (68 mg, 65%) as a colorless oil. TLC $R_f = 0.13$ (EtOAc/hexane, 2:1); MS (APCI, pos. 20 V) *m*/*z* 393 $(M+H)^+$; ^1H NMR (300 MHz, $CDCl_3$) δ 1.18 (d, $J = 6.6$ Hz, 3H), 1.29–1.51 (m, 12H), 1.80–2.08 (m, 4H), 2.19–2.35 (m, 1H), 2.54–2.73 (m, 1H), 3.09–3.25 (m, 1H), 3.38–3.61 (m, 6H), 3.99 (d, $J = 17.2$ Hz, 1H), 4.34–4.56 (m, 2H), 4.65 (d, $J = 17.2$ Hz, 1H).

According to the same procedures as described for the preparation of **79a** from **78**, compounds **79b**–**e** were prepared from **78**.

4.1.70. *tert*-Butyl (2*S*,3*S*,5*S*)-5-[(cyanomethyl)(ethyl)carbamoyl]-2-methyl-3-(1-piperidinylcarbonyl)-1-pyrrolidinecarboxylate (79b). Yield 54%. A colorless oil. TLC $R_f = 0.26$ (EtOAc/hexane, 2:1); MS (APCI, pos. 20 V) *m*/*z* 407 $(M+H)^+$; ^1H NMR (300 MHz, $CDCl_3$) δ 1.14–1.22 (m, 3H), 1.34 (t, $J = 7.2$ Hz, 3H), 1.39–1.48 (m, 9H), 1.49–1.78 (m, 6H), 2.16–2.33 (m, 1H), 2.59–2.75 (m, 1H), 3.17–3.31 (m, 1H), 3.32–3.82 (m, 6H), 3.98 (d, $J = 17.2$ Hz, 1H), 4.27–4.40 (m, 1H), 4.48 (dd, $J = 10.2$, 7.8 Hz, 1H), 4.65 (d, $J = 17.2$ Hz, 1H).

4.1.71. *tert*-Butyl (2*S*,3*S*,5*S*)-5-[(cyanomethyl)(ethyl)carbamoyl]-2-methyl-3-(4-morpholinylcarbonyl)-1-pyrrolidinecarboxylate (79c). Yield 65%. A colorless oil. TLC $R_f = 0.10$ (EtOAc/hexane, 2:1); MS (APCI, pos. 20 V) *m*/*z* 409 $(M+H)^+$; ^1H NMR (300 MHz, $CDCl_3$) δ 1.16–1.23 (m, 3H), 1.31–1.38 (m, 3H), 1.40–1.48 (m, 9H), 2.20–2.35 (m, 1H), 2.60–2.75 (m, 1H), 3.14–3.31 (m, 1H), 3.41–3.81 (m, 10H), 4.01 (d, $J = 17.2$ Hz, 1H), 4.25–4.39 (m, 1H), 4.49 (dd, $J = 10.3$, 7.9 Hz, 1H), 4.63 (d, $J = 17.2$ Hz, 1H).

4.1.72. *tert*-Butyl (2*S*,3*S*,5*S*)-5-[(cyanomethyl)(ethyl)carbamoyl]-3-(1,3-dihydro-2H-isoindol-2-ylcarbonyl)-2-methyl-1-pyrrolidinecarboxylate (79d). Yield 59%. A colorless oil. TLC $R_f = 0.26$ (EtOAc/hexane, 2:1); MS (APCI, pos. 20 V) *m*/*z* 441 $(M+H)^+$; ^1H NMR (300 MHz, $CDCl_3$) δ 1.23 (d, $J = 6.6$ Hz, 3H), 1.36 (t, $J = 7.1$ Hz, 3H), 1.41–1.51 (m, 9H), 2.27–2.41 (m, 1H), 2.62–2.79 (m, 1H), 3.22–3.36 (m, 1H), 3.55 (q, $J = 7.1$ Hz, 2H), 4.01 (d, $J = 17.0$ Hz, 1H), 4.49–4.60 (m, 1H), 4.66 (d, $J = 17.0$ Hz, 1H), 4.74–4.98 (m, 5H), 7.28–7.37 (m, 4H).

4.1.73. *tert*-Butyl (2*S*,3*S*,5*S*)-3-(benzylcarbamoyl)-5-[(cyanomethyl)(ethyl)carbamoyl]-2-methyl-1-pyrrolidinecarboxylate (79e). Yield 45%. A colorless oil. TLC $R_f = 0.26$ (EtOAc/hexane, 2:1); MS (APCI, pos. 20 V) *m*/*z* 429 $(M+H)^+$; ^1H NMR (300 MHz, $CDCl_3$) δ1.23 (d, $J = 6.6$ Hz, 3H), 1.34 (t, $J = 7.2$ Hz, 3H), 1.42 (s, 9H), 2.24–2.55 (m, 2H), 2.95–3.10 (m, 1H), 3.43–3.60 (m, 2H), 3.95–4.36 (m, 3H), 4.40–4.62 (m, 3H), 5.76–5.90 (m, 1H), 7.22–7.40 (m, 5H).

According to the same procedures as described for the preparation of **4b** from **30**, compounds **16**–**20** were prepared from **79a**–**79e**, respectively.

4.1.74. (2*S*,4*S*,5*S*)-*N*-(Cyanomethyl)-*N*-ethyl-5-methyl-4-(1-pyrrolidinylcarbonyl)-2-pyrrolidinecarboxamide 4-methylbenzenesulfonate (16). Yield 90%. A white powder. TLC $R_f = 0.62$ (CH_2Cl_2/MeOH, 5:1); MS (FAB, pos.) *m*/*z* 293 $(M+H)^+$; IR (KBr) 3432, 2247, 1660, 1632, 1469, 1459, 1189, 1123, 1035, 685 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6) δ 1.09–1.26 (m, 6H), 1.70–1.95 (m, 4H), 2.01–2.17 (m, 1H), 2.28 (s, 3H), 2.57–2.75 (m, 1H), 3.21–3.35 (m, 2H), 3.35–3.52 (m, 5H), 3.82–3.98 (m, 1H), 4.39 (d, $J = 18.0$ Hz, 1H), 4.50 (d, $J = 18.0$ Hz, 1H), 4.59–4.76 (m, 1H), 7.07–7.14 (m, 2H), 7.47 (d, $J = 8.1$ Hz, 2H), 8.03–8.21 (m, 1H), 9.55–9.74 (m, 1H); HRMS (FAB) calcd for $C_{16}H_{25}N_4O_5S$: 293.1978. Found: 293.1981.

4.1.75. (2*S*,4*S*,5*S*)-*N*-(Cyanomethyl)-*N*-ethyl-5-methyl-4-(1-piperidinylcarbonyl)-2-pyrrolidinecarboxamide 4-methylbenzenesulfonate (17). Yield 78%. A white powder. TLC $R_f = 0.63$ (CH_2Cl_2/MeOH, 5:1); MS (FAB, pos.) *m*/*z* 307 $(M+H)^+$; IR (KBr) 3429, 3367, 2925, 2251, 1659, 1631, 1468, 1250, 1124, 1035 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6) δ 1.09–1.24 (m, 6H), 1.35–1.66 (m, 6H), 2.04–2.18 (m, 1H), 2.28 (s, 3H), 2.54–2.68 (m, 1H), 3.32–3.94 (m, 8H), 4.38 (d, $J = 18.0$ Hz, 1H), 4.51 (d, $J = 18.0$ Hz, 1H), 4.59–4.76 (m, 1H), 7.07–7.14 (m, 2H), 7.47 (d, $J = 8.1$ Hz, 2H), 8.09–8.24 (m, 1H), 9.42–9.60 (m, 1H); HRMS (FAB) calcd for $C_{16}H_{27}N_4O_2$: 307.2134. Found: 307.2105.

4.1.76. (2*S*,4*S*,5*S*)-*N*-(Cyanomethyl)-*N*-ethyl-5-methyl-4-(4-morpholinylcarbonyl)-2-pyrrolidinecarboxamide 4-methylbenzenesulfonate (18). Yield 93%. A white powder. TLC $R_f = 0.55$ (CH_2Cl_2/MeOH, 5:1); MS (FAB, pos.) *m*/*z* 309 $(M+H)^+$; IR (KBr) 3433, 2923, 2852, 2251, 1659, 1633, 1469, 1035, 1011, 686 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6) δ 1.08–1.28 (m, 6H), 2.04-2.21 (m, 1H), 2.28 (s, 3H), 2.54–2.68 (m, 1H), 3.32–3.70 (m, 11H), 3.78–3.96 (m, 1H), 4.39 (d, $J = 18.0$ Hz, 1H), 4.51 (d, $J = 18.0$ Hz, 1H), 4.59–4.78 (m, 1H), 7.07–7.14 (m, 2H), 7.47 (d, $J = 7.9$ Hz, 2H), 8.11–8.30 (m, 1H), 9.47–9.67 (m, 1H); HRMS (FAB) calcd for $C_{19}H_{25}N_4O_2$: 309.1927. Found: 309.1919.

4.1.77. (2*S*,4*S*,5*S*)-*N*-(Cyanomethyl)-4-(1,3-dihydro-2*H*-isoindol-2-ylcarbonyl)-*N*-ethyl-5-methyl-2-pyrrolidinecarboxamide 4-methylbenzenesulfonate (19). Yield 85%. A white powder. TLC $R_f = 0.69$ (CH_2Cl_2/MeOH, 5:1); MS (FAB, pos.) *m*/*z* 341 $(M+H)^+$; IR (KBr) 3437, 2251, 1659, 1636, 1466, 1187, 1123, 1011, 685,

570 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6) δ 1.08-1.33 (m, 6H), 2.10-2.24 (m, 1H), 2.28 (s, 3H), 2.64–2.82 (m, 1H), 3.35–3.64 (m, 3H), 3.92–4.10 (m, 1H), 4.40 (d, J = 18.0 Hz, 1H), 4.52 (d, J = 18.0 Hz, 1H), 4.59–5.00 (m, 5H), 7.05–7.16 (m, 2H), 7.23–7.41 (m, 4H), 7.47 (d, J = 8.1 Hz, 2H), 8.09–8.29 (m, 1H), 9.61–9.80 (m, 1H); HRMS (FAB) calcd for $C_{16}H_{25}N_4O_8S$: 341.1978. Found: 341.1979.

4.1.78. (2*S*,4*S*,5*S*)-4-Benzylaminocarbonyl-*N*-(Cyanomethyl)-*N*-ethyl-5-methyl-2-pyrrolidinecarboxamide 4-methylbenzenesulfonate (20). Yield 99%. A white powder. TLC R_f = 0.57 (CH_2Cl_2/MeOH, 5:1); MS (FAB, pos.) m/z 329 $(M+H)^+$; IR (KBr) 3425, 3366, 2924, 2260, 1661, 1186, 1123, 1035, 1011, 685 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6) δ 1.09–1.26 (m, 6H), 1.99–2.15 (m, 1H), 2.28 (s, 3H), 2.60–2.78 (m, 1H), 3.10–3.22 (m, 1H), 3.36–3.50 (m, 2H), 3.75–3.91 (m, 1H), 4.18–4.35 (m, 2H), 4.39 (d, J = 18.0 Hz, 1H), 4.45–4.56 (m, J = 18.0 Hz, 1H), 4.58–4.76 (m, 1H), 7.06–7.14 (m, 2H), 7.20–7.37 (m, 5H), 7.47 (d, J = 8.2 Hz, 2H), 8.02–8.19 (m, 1H), 8.68–8.77 (m, 1H), 9.65–9.82 (m, 1H); HRMS (FAB) calcd for $C_{18}H_{25}N_4O_2$: 329.1978. Found: 329.199.

4.2. Biological method

4.2.1. Purification of human DPP-IV. Human DPP-IV was purified according to the published procedure with some modifications.[23] Briefly, the enzyme was prepared from pooled plasma obtained from healthy volunteers by ammonium sulfate precipitation (50–70%). After extensive dialysis against 25 mM Tris–HCl (pH 7.4), the material was mixed with DEAE cellulose, DE52 (Whatman Chemical Separation, Inc., USA) for 60 min, and eluted with buffer containing 100 mM NaCl. Fractions of 10 mL were collected, and the fraction with maximal DPP-IV activity was dialyzed against 25 mM MES–NaOH (pH 6.0). DPP-IV-containing fractions were detected by the ability to hydrolyze Gly-Pro-7-amido-4-methyl-coumarin (Gly-Pro-AMC) (Sigma–Aldrich, USA) using the standard method described below. The DE52 elute was loaded onto a SP Sepharose Fast Flow column (GE Healthcare, Sweden), and the flow-through fraction containing DPP-IV was then applied to a DEAE cellulose column (Whatman DE52). Bound proteins were eluted with 25 mM Tris–HCl (pH 7.8) containing 150 mM NaCl. Fractions of 10 mL were collected, and the fraction with maximum DPP-IV activity was concentrated using polyethylene glycol 20000 (PEG20000). The concentrated material was applied to a Sephacryl S-300 High Resolution 26/60 column (GE Healthcare, Sweden) and eluted at a flow rate of 0.1 mL/min. Fractions of 1 mL were collected, and the fractions containing DPP-IV activity were pooled.

4.2.2. Enzyme assays. Enzymatic activity was determined at 37 °C by the cleavage rate of a substrate, Gly-Pro-AMC (30 μM) (Sigma–Aldrich, USA).[24] Briefly, 10 μL of DPP-IV solution was added to each well of a 96-well flat-bottomed microtiter plate, followed by the addition of 50 μL of 60 μM Gly-Pro-AMC, 10 μL of 500 mM Tris–HCl (pH 7.4), 20 μL of distilled water, and 10 μL of a test compound. Then the change of fluorescence was monitored at 37 °C using a spectrofluorometer (excitation at 355 nm/emission at 460 nm) (fmax, Molecular Devices, USA). The initial rate of DPP-IV activity was calculated over the first 15 min of the reaction and defined as the rate of increase in the fluorescence intensity (arbitrary units 1 mL) under these conditions. The percent inhibition was calculated relative to the addition of the solvent alone and IC_{50} values were determined by logistic regression analysis.

4.2.3. DPP-IV inhibition in rats. Male Sprague–Dawley (SD) rats were purchased from Charles River Laboratories, Japan. The rats were housed in an air-conditioned animal room with controlled temperature (24 ± 2 °C), humidity (55 ± 5%), and lighting (12:12 h light/dark cycle), and were provided with standard pellet food for rodents (CRF-1, Oriental Yeast, Japan) and water ad libitum. All procedures were conducted according to the ONO Pharmaceutical Animal Care Committee guidelines. After fasting for at least 8 h, male SD rats (6–7 weeks old) were orally administered a test compound dissolved in 0.5% methyl cellulose as a single dose of 0.3 mg/kg. Blood samples were collected from the jugular vein before administration, and 0.25, 0.5, 1, 2, 4, 6, and 9 h after administration. Each blood sample was immediately centrifuged to obtain plasma and the DPP-IV activity was determined. Briefly, 50 μL of plasma was added to each well of a 96-well flat-bottomed microtiter plate, followed by the addition of 50 μL of 60 μM substrate. Then the initial rate of DPPIV activity was measured using the method described above, and the percent inhibition relative to basal DPP-IV activity was calculated.

4.2.4. Multiple oral glucose tolerance tests in rats. The effect of inhibitor **11** on the outcome of multiple oral glucose tolerance tests was assessed in male SD rats (364–473 g). The rats were fasted for at least 20 h before being studied and then dosed orally with the vehicle (0.5% methyl cellulose) or with compound **11** (1 mg/kg) at −0.5 h. A blood sample (75 μl) was collected from the tail vein into heparinized tubes at −0.08 h. Glucose (1 g/kg) was administered orally at 0 and 6 h. Additional blood samples (75 μl) were collected at 0.17, 0.5, 1, 2, 6, 6.17, 6.5, 7, and 8 h after the first glucose load. Plasma was obtained from each sample by centrifugation and was stored at −80 °C until measurement of the glucose level with a glucose oxidase peroxidase dye system (Diacolor GC, Toyobo, Japan).

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