concentration-percent cell-survival curves and were defined as the concentrations of phenoxazines required for 50% reduction in colonies compared to controls (Table II).

Effect of N-Substituted Phenoxazines on in Vitro Cytotoxicity of VLB and VCR. Cells were treated with graded concentrations of VCR and VLB in the absence or presence of nontoxic concentrations (Table III) of compounds 1, 3, 4, 11, or 18. The plates were then transferred to a CO_2 incubator and, after further incubation for 7 days at 37 °C, colonies were enumerated as described.

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Synthesis and Biological Activity of Ketomethylene Pseudopeptide Analogues as Thrombin Inhibitors[1]

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Ketomethylene pseudopeptide analogues Aa-Pro-Argψ($COCH_2$)Gly-pip, 1, where Aa are D- or L-amino acids (Dpa, β,β-diphenylalanine; αNal, α-naphthylalanine; βNal, β-naphthylalanine; Fgl, fluorenylglycine) with highly lipophilic side chains and ψ($COCH_2$) is a ketomethylene pseudopeptide bond, have been synthesized through a modified Dakin-West reaction under very mild conditions with a high yield using tripeptide 4 with a labile functional group directly on the side chain. Their enzymatic assay of thrombin inhibition has been carried out. The structure-activity relationship study indicated that a lipophilic side chain on the amino acid in the P_3 position is very important for binding to the apolar site of thrombin. Compound **1a** with D-Dpa at the P_3 position has a K_i of 0.2 μM and it doubles thrombin clotting time at only 3 times higher concentration. These values are about 7 times better than those of the corresponding D-Phe analogues. Furthermore, **1a** shows poor inhibitory activity against plasmin, factor Xa, urokinase, and kallikrein. Preliminary in vivo testing (3-4-kg rabbit as the animal model) shows no observable side effect (change of blood pressure and accumulation of blood platelet in lungs) at a dose of 1 mg/kg.

Interest in the design of synthetic inhibitors of serine proteases and especially of thrombin has grown enormously during the last few years. One type of such inhibitors is the ketomethylene pseudopeptide analogues, in which the -NH- group of the scissile P_1-P_1' bond, corresponding to the natural substrate, has been replaced by a methylene group. This type of peptide inhibitor has several advantages. Firstly, the ketomethylene bond is resistant to enzymatic degradation. Secondly, the keto group can possibly form a tetrahedral semi-ketal with the active site serine hydroxyl. Thirdly, the amino acid sequence on the carbonyl side of the ketomethylene bond can add binding affinity to the inhibitor.

The peptide sequence D-Phe-Pro-Arg, imitating the natural substrate of thrombin, has been widely used as a basis for inhibitors and substrates of thrombin.[2-6] The ketomethylene peptide analogue D-Phe-Pro-Argψ-($COCH_2$)Gly-pip, synthesized by Szelke and Jones,[6] showed good inhibitory activity toward thrombin.[7]

In the course of our studies on thrombin peptide inhibitors, it is found that a lipophilic side chain on the amino acid in the P_3 position is very important for binding to the apolar site of thrombin. We here report the synthesis and antithrombin effect of inhibitors with the structure shown in formula 1 (Scheme I), where D-Phe in the sequence D-Phe-Pro-Arg has been replaced by some unnatural, aromatic amino acids.

To introduce the ketomethylene moiety into the compound, Szelke and Jones[6] used a Dakin-West reaction[8] with N^{α}-formyl-protected amino acid as the starting material. This method gave us a very poor yield (1%). The benzene ring of an N^{α}-benzoyl amino acid activatives the oxazolone intermediate. We obtained a good yield (58%) by this method, but the very harsh conditions (HCl/$AcOH/H_2O$, 120 °C, 20 h)[9] needed to remove the benzoyl protecting group gave a very poor yield in this step. These hydrolysis conditions will limit the use of this reaction in the case of amino acids with labile functional groups on the side chains. The trifluoromethyl ketone analogue of

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(1) Abbreviations: Aa, α-amino acid; Dpa, β,β-diphenylalanine; αNal, α-naphthylalanine; βNal, β-naphthylalanine; Fgl, fluorenylglycine; ψ($COCH_2$), ketomethylene pseudopeptide bond; MMS, monomethyl succinate; Py, pyridine; TEA, triethylamine; pip, piperidine; Mtr, (4-methoxy-2,3,6-trimethylphenyl)sulfonyl; HOSu, *N*-hydroxysuccinimide; DCC, 1,3-dicyclohexylcarbodiimine; TFA, trifluoroacetic acid; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride.

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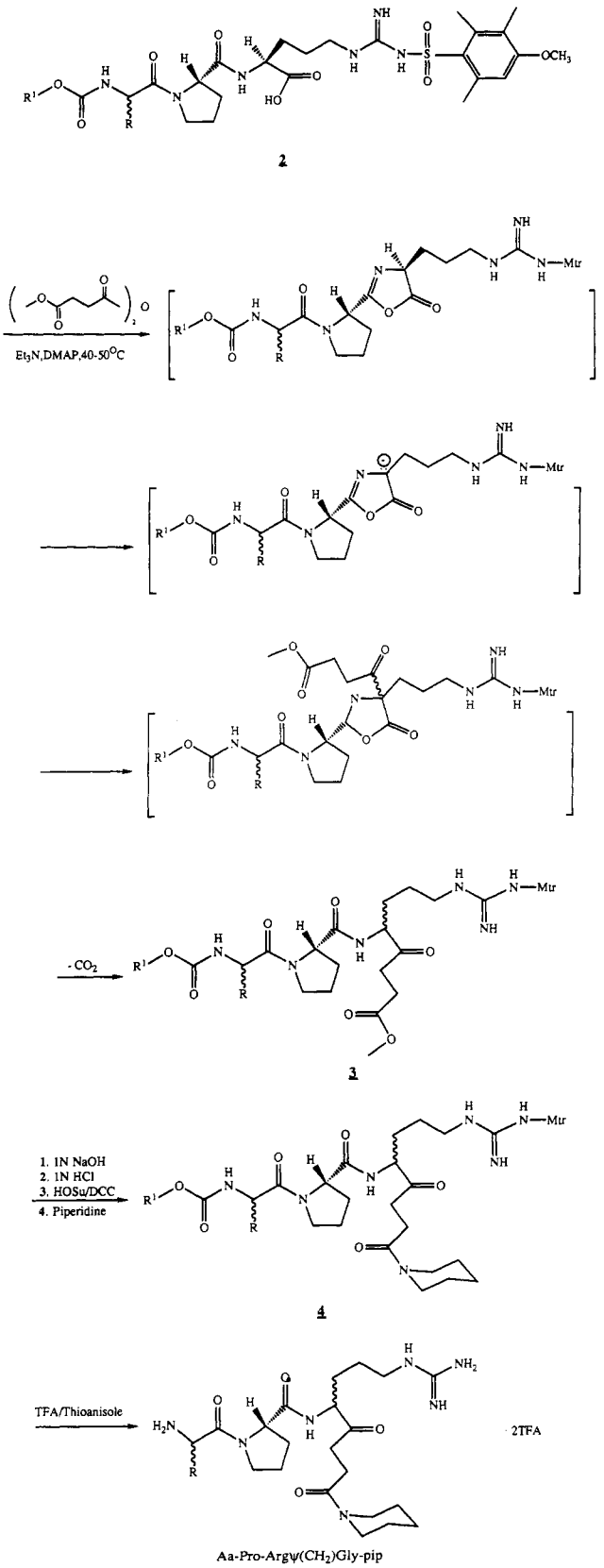
Scheme I



2

3

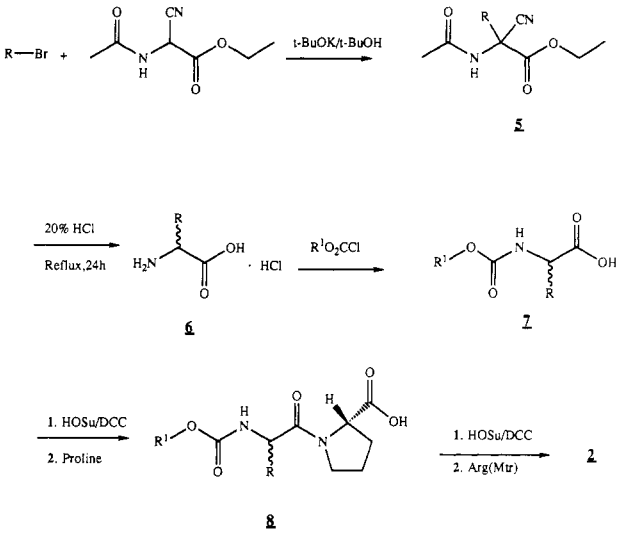
4

Aa-Pro-Argψ(CH_2)Gly-pip

1

	R	Aa
1a	Diphenylmethyl	D-Dpa
1b	Diphenylmethyl	L-Dpa
1c	Diphenylmethyl	D,L-Dpa
1d	α-Naphthylmethyl	D,L-αNal
1e	β-Naphthylmethyl	D,L-βNal
1f	β-Naphthylmethyl	D-βNal
1g	Fluorenyl	D,L-Fgl

Scheme II



5 **6** **7** **8**

D-Phe-Pro-Arg-OH was synthesized by Neises and Turnus[10] with *N,N*-dibenzoylornithine as starting material through the Dakin–West reaction in a very complicated way due to the difficulty in removing the benzoyl groups. Arginine can not be used directly because of the drastic conditions used. For the preparation of a bifunctional inhibitor, D-Phe^{45}-Argψ($COCH_2$)CH_2CO^{47}hirudin$^{45-65}$, Ac-D-Phe-Pro-Argψ($COCH_2$)Gly-OH was synthesized from the reaction of N^{α}-*t*-Boc-N^{ω}-tosylarginine *N,O*-dimethyl hydroxamate with Grinard reagent following an oxidation reaction.[11]

Scheme I summarizes a facile and direct route based on a modified Dakin–West reaction[12] for the synthesis of **1**. Instead of formyl or benzoyl groups, we use the dipeptide R^1OCO-Aa-Pro-OH (**8**) (R^1 = *t*-Bu or Bzl) itself as the protecting group. Thus the tripeptide **2** was heated with the anhydride of MMS, TEA, and DMAP at 40–50 °C for 1 h and then was treated with 4% sodium hydrogen carbonate. The solution was stirred for an additional 30 min. Product **3** was isolated on a silica gel column with 2% methanol in chloroform as eluent. It was hydrolyzed, activated (HOSu,DCC), and then coupled with piperidine. Product **4** was separated on a silica gel column (conditions as above). The Z (or Boc) and Mtr protecting groups were removed by treatment with 9:1 TFA/thioanisole, (room temperature, 3 h).[13] After precipitation and washing with ether pure product **1** was obtained as a white solid.

The unnatural, aromatic amino acids have been synthesized through the alkylation of ethyl acetamidocyanoacetate with RBr (R = diphenylmethyl, α-naphthylmethyl, β-naphthylmethyl, and fluorenyl) in the presence of potassium *tert*-butoxide (Scheme II) followed by acidic hydrolysis. The dipeptide **8** and tripeptide **2** were prepared by standard methods.

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Table I. Differences in Physical Date of Dipeptide 8a and 8b

compd	HPLC, min	^{1}H NMR δ, ppm, NH	$[\alpha]^{25}_D$, deg	mp, °C
8a	16.9	5.66 (d, J = 9 Hz)	−139.0 (c 0.7, EtOH)	180–183
8a + 8b	14.5, 16.7	5.61 (d, J = 9 Hz) 5.55 (d, J = 9 Hz)		126–130
8b	14.6	5.55 (d, J = 9 Hz)	−21.0 (c 1.0, EtOH)	128–131

Table II. Thrombin Inhibitory Activity of Compound 1

compd	K_i, μM	TT,[a] μM	APTT,[b] μM
Szelke[c]	1.3	4.9	
1a	0.2	0.65	8.0
1b	1.7	7.5	100
1c	0.6		45
1d	13.5		
1e	11.6	90	
1f	2.28		
1g		50	

[a] Concentration needed to double the human α-thrombin-induced clotting time of human plasma (control, 25 s). [b] Concentration needed to double the activated partial thromboplastin time of human plasma (auto-APTT reagent, general Diagnostic; control clotting time, 30 s). [c] D-Phe-Pro-Argψ(COCH$_2$)Gly-pip (data from ref 7).

The diastereomers Z-D-Dpa-Pro-OH (8a) and Z-L-Dpa-Pro-OH (8b) have been separated through fractioned recrystallization from ethyl acetate. One diastereomer comes out first as white crystals. A second crop, a mixture of diastereomers, was obtained by concentration of the mother liquid. The other isomer was obtained by addition of petroleum ether (bp: 60–80 °C). It was found that the first crop had Dpa in the D-configuration by crystallographic analysis, which will be reported in detail elsewhere. Both isomers were characterized by HPLC, ^{1}H NMR, and $[\alpha]^{25}_D$ (Table I), which indicate the high optical purity of both isomers (95% ee).

Results and Discussion

Table II shows the results of the in vitro inhibitory activity of compound 1. Our earlier results have shown that in general tripeptides with the P_3 amino acid in the D-form have higher affinity to thrombin than the corresponding peptide with this amino acid in the L-form.[2] Compound 1a from the first crop (8a) with D-Dpa is about 8 times more potent than 1b from the third crop (8b), and about 3 times more potent than 1c from a fully racemic starting material 2c. Compound 1f synthesized from D-βNal is 5 times more active than compound 1e from racemic 2e. It is also shown in the table that substitution with D,L-αNal or D,L-Fgl gives less potent thrombin inhibitors.

The Dakin–West reaction gives no stereoselectivity at the α-carbon of the arginine in the acylation reaction through a oxazalone intermediate (Scheme I). The evidence for racemization was obtained by HPLC analysis of both 1a and 1b as well as 1f, which clearly indicated the existence of two isomers (retention times: 1a, 14.73, 16.45 min; 1b, 14.71, 16.63 min; 1f, 11.60, 12.41 min). It is interesting to investigate which isomer is the most active thrombin inhibitor.

The crystal structure of PPACK-thrombin[14] indicates that the loop Tyr-Pro-Pro-Trp (47–50) creates with Trp^{227}(215), Leu^{96}(99), and Ile^{179}(174) a double hydropholic pocket, a "D-pocket", and a "P-pocket", respectively. PPACK binds with D-Phe in the D-pocket and Pro in the P-pocket. The inhibitor Aa-Pro-Argψ(COCH$_2$)Gly-pip (1) might bind to thrombin in a similar mode with Aa in the D-pocket (S_3), Pro in the P-pocket (S_2), and the Arg side chain in the recognition pocket (S_1). Compound 1f with D-βNal has a better K_i than 1e with racemic βNal because the D-pocket prefers a D-amino acid in the P_3 position. In the same way, the D-pocket would prefer D-Dpa to L-Dpa. Compound 1a has a K_i of 0.2 μM and it doubles thrombin clotting time at only 3 times higher concentration. These values are about 7 times better than those of the corresponding D-Phe analogues.[6] This might be explained by the extra phenyl group in Dpa producing higher affinity to the D-pocket, compared to D-Phe. The amino acid βNal gives inhibitors roughly comparable to the Phe inhibitors while Fgl is not a good substitute for Phe. The rigid aromatic ring system in Fgl seems harder to accommodate, compared to the flexible ring in Dpa, preventing a good interaction with the thrombin binding site.

Table III. Selectivity of Compound 1a for Thrombin Inhibition

	thrombin	plasmin	factor Xa	urokinase	kallikrein
K_i, μM	0.2	157	149	494	199

Table III shows the selectivity of the inhibitor 1a. It is very poor inhibitor for plasmin, urokinase, kallikrein, and factor Xa. Thrombin has an extra loop, the "Try-Pro-Pro-Trp loop", not present in trypsin.[14] The high specificity of 1a for thrombin thus could be explained by the binding of Pro to the P-pocket and D-Dpa to the D-pocket. Preliminary in vivo testing (3–4-kg rabbit as the animal model) shows no observable side effect (change of blood pressure and accumulation of blood platelet in lungs) at a dose of 1 mg/kg.

Experimental Section

Melting points were measured on a Stuart capillary melting point apparatus and are uncorrected. TLC was carried out on precoated silica plates (Merk, F 254) in the following systems: A, chloroform–methanol (9:1); B, 1-butanol–acetic acid-ethyl acetate–water (1:1:1:1); C, ethyl acetate–ethanol–acetic acid (2:1:0.1); D, chloroform–methanol–acetic acid (95:5:3). The plates were visualized with the following reagent sprays: (I) ninhydrin (BDH), 0.1% in ethanol; (II) chlorine, dicarboxidine; (III) UV (254 nm); (III) iodine vapors. HPLC analysis was carried out on a LKB liquid chromatograph, using a LKB C_2/C_{18} Superpac PepS column (4.0 × 250 mm) and the following conditions: eluent, A = water containing 0.1% TFA; B = acetonitrile containing 0.1% TFA; gradient (in 25 min), (1) 50–90%, (2) 35–70%, (3) 10–50%; flow rate, 1.0 mL/min; detection, UV absorbance at 210 nm. ^{1}H and ^{13}C NMR spectra were taken on a Bruker WM 250 instrument. Mass spectrometry was performed using a fast bombardment (FAB) ion source. Chromatography was carried on Merck flash silica gel.

EDC was purchased from Navobiochem. D-βNal was purchased from Bachem [$[\alpha]_D$ = +16.0° (c = 0.4, 50% AcOH)].

Preparation of the Anhydride of MMS. A solution of succinic anhydride (10.0 g, 0.1 mol), methanol (3.2 g, 0.1 mol), and DMAP (2 mg) in 50 mL of toluene was refluxing for 3.5 h and then cooled to room temperature. After evaporation of the solvent, crystals formed. The monomethyl succinate (MMS) was collected by filtration and washed with petroleum ether (12.5 g, 94%). Mp: 52–54 °C.

The MMS (0.32 g, 2.4 mmol) was dissolved in 20 mL of CH_2Cl_2 and 0.23 g of EDC (1.2 mmol) was added. The reaction was stirred for 20 min and then extracted with 2 × 3 mL of ice-cold water, 3 mL of ice-cold 5% $NaHCO_3$, and 3 mL of ice-cold water. The organic layer was dried ($MgSO_4$) and filtered into a reaction flask for the following Dakin–West reaction, and the solvent was evaporated in vacuo.

Preparation of D,L-Dpa (6a). To a solution of potassium *tert*-butoxide (6.75 g, 0.06 mol) in *tert*-butyl alcohol (350 mL) was

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added, at room temperature under argon, ethyl acetamidocyanoacetate (10 g, 0.059 mol). After the solution had become clear bromodiphenylmethane (14.55 g, 0.059 mol) was added. The mixture was stirred at 20 °C for 24 h and then evaporated under reduced pressure. The solid residue was treated with ethyl acetate (500 mL) and water (175 mL). The organic phase was dried (Na_2SO_4) and concentrated to give yellow crystals. The crystals were washed repeatedly with ether and dried to give ethyl acetamido(diphenylmethyl)cyanoacetate (**5a**) (11.61 g, 58%). Mp: 181–185 °C.

Compound **5a** was mixed with hydrochloric acid (20%) and refluxed for 30 h. The reaction mixture was allowed to cool, and the crystals were collected, washed (ether), and dried to give **6a** as a salt of hydrogen chloride (7.82 g, 82%). R_f (solvent B): 0.67. ^{1}H NMR δ (DMSO-d_6): 7.54–7.11 (10 H, m, $2C_6H_5$), 4.79 (1 H, d, αCH), 4.36 (1 H, d, βCH).

The following compounds **6** were synthesized as above.

D,L-αNal (6b) (1.08 g, 36%). Mp: 198–201 °C. R_f(D): 0.10 ^{1}H NMR δ (DMSO-d_6): 8.10–7.25 (7 H, m, $C_{10}H_7$), 3.47 (2 H, m, CH_2).

D,L-βNal (6c) (2.89 g, 79%). ^{1}H NMR δ (DMSO-d_6): 7.79–7.27 (7 H, m, $C_{10}H_7$), 4.48 (1 H, m, αCH), 3.35 (2 H, d, CH_2).

D,L-Fgl (6d) (4.76 g, 72%). ^{1}H NMR δ (D_2O): 7.86–7.37 (8 H, m, $C_{13}H_8$), 5.00 (1 H, m, αCH), 4.66 (1 H, d, βCH).

Preparation of Boc-D,L-Dpa (7a). To a solution of **6a** (2.78 g, 10 mmol) in a mixture of dioxane (20 mL), water (10 mL), and 1 N NaOH (20 mL), cooled to 0 °C and stirred, was added di-*tert*-butylpyrocarbonate (2.4 g, 11 mmol), stirring was continued at room temperature for 2 h. The solution was concentrated in vacuo to 20 mL, cooled in an ice–water bath, covered with a layer of ethyl acetate (30 mL), and acidified to pH 2–3 with dilute solution of $KHSO_4$. The aqueous layer was extracted with ethyl acetate, and the combined organic layers were dried ($MgSO_4$) and evaporated to dryness to give **7a** as a solid (3.38 g, 90%). Mp: 127–129 °C. ^{1}H NMR δ (DMSO-d_6): 7.27 (19 H, m, $2C_6H_5$), 5.07 (1 H, d, NH), 4.86 (1 H, d, αCH), 4.49 (1 H, d, βCH), 1.36 (9 H, s, t-C_4H_9).

The following compounds **7** were synthesized as above.

Boc-D,L-αNal (7c) (1.23 g, 82%). Mp: 148–151 °C. R_f(D): 0.54. ^{1}H NMR δ (DMSO-d_6): 8.10–7.25 (7 H, m, $C_{10}H_7$), 5.12 (1 H, d, NH), 4.83 (1 H, m, CH), 3.38 (2 H, d, CH_2), 1.36 (9 H, s, t-C_4H_9).

Boc-D,L-βNal (7d) (1.92 g, 61%). ^{1}H NMR δ ($CDCl_3$): 7.75–7.25 (7 H, m, $C_{10}H_7$) 5.23 (1 H, d, NH), 4.74 (1 H, m, CH), 3.31 (2 H, d, CH_2), 1.36 (9 H, s, t-C_4H_9).

Boc-D,L-Fgl (7f) (2.90 g, 85%). ^{1}H NMR δ ($CDCl_3$): 7.74–7.23 (8 H, m, $C_{13}H_8$), 5.62 (1 H, d, NH), 5.01 (1 H, m, αCH), 4.08 (1 H, d, βCH), 1.37 (9 H, s, t-C_4H_9).

Z-D,L-Dpa (7b). To a solution of **6a** (2.45 g, 10 mmol) in water (10 mL) and dioxane (10 mL) in the presence of triethylamine (1.7 mL, 12.2 mmol) was added *N*-[(benzyloxycarbonyl)oxy]-succinimide (2.54 g, 10.2 mmol) at room temperature and the mixture was stirred overnight. The reaction mixture was poured into 50 mL of water and the product was extracted with 100 mL of chloroform, washed with 1 N HCl then water, and dried (Na_2SO_4). After removal of the chloroform the residue was crystallized from ethyl acetate and petroleum ether (bp: 40–60 °C) (3.40 g, 90%) as white solid. Mp: 214–217 °C. ^{1}H NMR δ (DMSO-d_6): 7.32–7.12 (10 H, m, $2C_6H_5$), 5.56 (1 H, d, NH), 5.27 (1 H, d, αCH), 5.02 (2 H, s, OCH_2), 4.41 (1 H, d, βCH).

Z-D-βNal (7e). Z-OSu was used (1.15 g, 95%). ^{1}H NMR δ ($CDCl_3$): 7.85–7.40 (7 H, m, $C_{10}H_7$), 7.29 (5 H, s, C_6H_5), 5.20 (1 H, d, NH), 5.08 (2 H, s, OCH_2), 4.77 (1 H, m, CH), 3.32 (2 H, d, CH_2).

Preparation of Boc-D,L-Dpa-ProOH (8c). A solution of **7a** (3.41 g, 10 mmol) and *N*-hydroxysuccinimide (1.15 g, 10 mmol) in dry 1,2-dimethoxyethane (30 mL) was cooled in an ice–water bath and DCC (2.06 g, 10 mmol) was added with stirring. Further stirring was continued for 4 h. The formed DCU was filtrated off and the solvent was evaporated in vacuo. The activate product was obtained through recrystallization from dichloromethane and petroleum ether (bp: 60–80 °C) (4.15 g, 94%). Mp: 185–187 °C.

To a solution of proline (0.78 g, 6.75 mmol) and sodium hydrogen carbonate (0.57 g, 6.75 mmol) in H_2O (10 mL) was added Boc-D,L-Dpa-OSu (1.97 g, 4.5 mmol) in 10 mL of 1,2-dimethoxyethane at room temperature with stirring. After 3 h, the organic solvent was evaporated. The aqueous solution was acidified with 1 N HCl to pH 2; a white solid formed and was collected with filtration, washed with ether, and dried (1.82 g, 92%). Mp: 94–96 °C. ^{1}H NMR δ ($CDCl_3$): 7.37–7.17 (10 H, m, $2C_6H_5$), 5.12 (1 H, m, NH), 4.38 (1 H, dd, Dpa αCH), 4.16 (1 H, d, Dpa βCH), 3.85 (1 H, m, Pro CH), 1.35 (9 H, s, t-C_4H_9).

Z-D,L-Dpa-ProOH (8a + 8b) (1.98 g, 93%). Mp: 126–130 °C.

Z-D-Dpa-ProOH (8a) (0.70 g). Mp: 180–183 °C (obtained by recrystallization from ethyl acetate). HPLC (2): 16.9 min. ^{1}H NMR δ ($CDCl_3$): 7.38–7.17 (15 H, m, $3C_6H_5$), 5.66 (1 H, d, NH), 5.26 (1 H, dd, Dpa αCH), 5.00 (2 H, m, OCH_2), 4.40 (1 H, d, Dpa βCH), 3.81 (1 H, t, Pro CH), 2.80 (2 H, m, NCH_2), 2.10–1.10 (4 H, m, 2 CH_2). MS m/z (FAB): 473 (M + H), 495 (M + Na).

Z-L-Dpa-ProOH (8b) (0.54 g). Mp: 128–131 °C (obtained by recrystallization from ethyl acetate–petroleum ether). HPLC (2): 14.6 min. ^{1}H NMR δ ($CDCl_3$): 7.43–7.10 (15 H, m, $3C_6H_5$), 5.55 (1 H, d, NH), 5.23 (1 H, dd, Dpa αCH), 5.04 (2 H, m, OCH_2), 4.45 (1 H, d, Dpa βCH), 3.78 (1 H, m, Pro CH), 2.90 (2 H, m, NCH_2), 2.25–1.10 (4 H, m, 2 CH_2). MS m/z (FAB): 473 (M + H), 495 (M + Na).

Boc-D,L-αNal-ProOH (8d) (1.35 g, 91%). Mp: 70–78 °C. R_f(D): 0.58. ^{1}H NMR δ ($CDCl_3$): 8.07–7.30 (7 H, m, $C_{10}H_7$), 5.24 (1 H, m, NH), 4.85 (1 H, m, Nal CH), 3.75 (1 H, m, Pro CH), 347 (2 H, m, Nal CH_2), 1.36 (9 H, s, t-C_4H_9).

Boc-D,L-βNal-ProOH (8e) (0.5 g, 89%). ^{1}H NMR δ ($CDCl_3$): 7.79–7.25 (7 H, m, $C_{10}H_7$), 4.56 (1 H, Nal CH), 3.66 (2 H, m, Nal CH_2), 1.31 (9 H, s, t-C_4H_9).

Z-D-βNal-ProOH (8f) (1.14 g, 89%). HPLC (1): 11.3 min. ^{1}H NMR δ ($CDCl_3$): 7.85–7.40 (7 H, m, $C_{10}H_7$), 7.29 (5 H, s, C_6H_5), 5.21 (1 H, d, NH), 5.08 (2 H, m, OCH_2), 4.77 (1 H, m, Nal CH), 3.79 (1 H, m, Pro CH), 3.30 (2 H, m, Nal CH_2).

Boc-D,L-Fgl-ProOH (8g) (2.01 g, 92%). HPLC (1): 6.5, 7.1 min. ^{1}H NMR δ ($CDCl_3$): 7.74–7.23 (8 H, m, $C_{13}H_8$), 5.89 (1 H, m, NH), 5.03 (1 H, m, Fgl αCH), 4.11 (1 H, m, Fgl βCH), 3.75 (1 H, m, Pro CH), 1.37 (9 H, s, t-C_4H_9).

Preparation of Boc-D,L-Dpa-Pro-Arg(Mtr)OH (2c). To a solution of **8c** (0.44 g, 1.0 mmol) and HOSu (0.12 g, 1.0 mmol) in 20 mL of 1,2-dimethoxyethane was added DCC (0.21 g, 1.0 mmol) with cooling over an ice–water bath, and then stirring was continued at room temperature for 3 h. The DCU was filtered off and the solvent was evaporated to give an oily crude activate dipeptide. To a solution of H-Arg(Mtr)OH (0.42 g, 1.1 mmol) and Et_3N (1.1 mmol) in 30 mL of DMF was added the solution of Boc-D,L-Dpa-Pro-OSu obtained above in 15 mL of 1,2-dimethoxyethane with cooling over an ice–water bath. The reaction mixture was stirred at room temperature overnight. The solvent was evaporated in vacuo. The residue was dissolved in methanol and 20 mL of water. The solution was acidified with 1 N HCl to pH 2. Methanol was removed in vacuo to give a white precipitate, which was collected through filtration and dried in vacuo (0.70 g, 88%). ^{1}H NMR δ ($CDCl_3$): 7.34–7.20 (10 H, m, $2C_6H_5$), 6.51 (1 H, s, CH), 6.37 (3 H, b, 3 NH), 5.42 (1 H, d, NH), 5.20 (1 H, d, NH), 4.81 (1 H, m, Dpa αCH), 4.35 (1 H, d, Dpa βCH), 4.20 (1 H, d, Arg CH), 3.81 (3 H, s, OCH_3), 3.75 (1 H, m, Pro CH), 2.68 (3 H, d, CH_3), 2.60 (3 H, d, CH_3), 2.11 (3 H, s, CH_3), 1.28 (9 H, d, t-C_4H_9), 3.52–1.10 (12 H, m, 6 CH_2).

Z-D-Dpa-Pro-Arg(Mtr)OH (2a) (0.77 g, 91%). ^{1}H NMR δ ($CDCl_3$): 7.47 (1 H, d, NH), 7.33–7.18 (15 H, m, $3C_6H_5$), 6.48 (1 H, s, CH), 6.32 (3 H, b, 3 NH), 5.83 (1 H, d, NH), 5.02 (2 H, m, OCH_2), 4.83 (1 H, d, Dpa αCH), 4.35 (1 H, d, Dpa βCH), 4.19 (1 H, d, Arg CH), 3.78 (3 H, s, OCH_3), 3.74 (1 H, m, Pro CH), 2.65 (3 H, s, CH_3), 2.57 (3 H, s, CH_3), 2.08 (3 H, s, CH_3), 3.55–1.00 (12 H, m, 6 CH_2).

Z-L-Dpa-Pro-Arg(Mtr)OH (2b) (0.82 g, 95%). ^{1}H NMR δ ($CDCl_3$): 7.34–7.20 (15 H, m, $3C_6H_5$), 6.48 (1 H, s, CH), 5.78 (1 H, d, NH), 5.02 (2 H, m, OCH_2), 4.80 (1 H, d, Dpa αCH), 4.38 (1 H, d, Dpa βCH), 4.19 (1 H, d, Arg CH), 3.78 (3 H, s, OCH_3), 3.74 (1 H, m, Pro CH), 2.65 (3 H, s, CH_3), 2.56 (3 H, s, CH_3), 2.09 (3 H, s, CH_3), 3.55–1.10 (12 H, m, 6 CH_2).

Boc-D,L-αNal-Pro-Arg(Mtr)-OH (2d) (0.72 g, 92%). R_f (C): 0.51. ^{1}H NMR δ ($CDCl_3$): 8.07–7.30 (7 H, m, $C_{10}H_7$), 6.48 (1 H, d, CH), 6.40 (3 H, b, 3 NH), 4.64 (1 H, m, Nal CH), 3.82 (1 H, m, Arg CH), 3.79 (3 H, s, OCH_3), 3.74 (1 H, m, Pro CH), 3.49 (2 H, m, Nal CH_2), 2.67 (3 H, s, CH_3), 2.59 (3 H, s, CH_3), 2.10 (3 H, s, CH_3), 1.39 (9 H, m, t-C_4H_9).

Boc-D,L-βNal-Pro-Arg(Mtr)-OH (2e) (0.29 g, 88%). ^{1}H NMR δ ($CDCl_3$): 7.75–7.25 (7 H, m, $C_{10}H_7$), 6.51 (1 H, s, CH), 6.47 (3 H, b, 3 NH), 4.75 (1 H, m, Nal CH), 4.21 (1 H, d, Arg CH), 3.81 (3 H, s, OCH_3), 3.74 (1 H, m, Pro CH), 3.47 (2 H, m, Nal CH_2), 2.63 (3 H, m, CH_3), 2.56 (3 H, m, CH_3), 2.09 (3 H, m, CH_3), 1.37 (9 H, m, t-C_4H_9), 3.55–1.10 (12 H, m, 6 CH_2). MS m/z (FAB): 804 (M + Na).

Z-D-βNal-Pro-Arg(Mtr)-OH (2f) (0.40 g, 97%). ^{1}H NMR δ ($CDCl_3$): 7.86–7.41 (7 H, m, $C_{10}H_7$), 7.29 (5 H, s, C_6H_5), 6.48 (1 H, s, CH), 5.02 (2 H, m, OCH_2), 4.76 (1 H, m, Nal CH), 4.21 (1 H, d, Arg CH), 3.75 (3 H, s, OCH_3), 3.74 (1 H, m, Pro CH), 3.46 (2 H, m, Nal CH_2), 2.65 (3 H, s, CH_3), 2.57 (3 H, s, CH_3), 2.08 (3 H, s, CH_3), 3.50–1.00 (12 H, m, 6 CH_2).

Boc-D,L-Fgl-Pro-Arg(Mtr)-OH (2g) (0.71 g, 88%). R_f (C): 0.61. ^{1}H NMR δ ($CDCl_3$): 7.74–7.23 (8 H, m, $C_{13}H_8$), 6.51 (1 H, s, CH), 4.94 (1 H, m, Fgl αCH), 4.23 (1 H, m, Arg CH), 4.11 (1 H, m, Fgl βCH), 3.78 (3 H, s, OCH_3), 3.75 (1 H, m, Pro CH), 2.64 (3 H, m, CH_3), 2.56 (3 H, m, CH_3), 2.10 (3 H, m, CH_3), 1.38 (9 H, m, t-C_4H_9), 3.55–1.00 (12 H, m, 6 CH_2).

Preparation of Boc-D,L-Dpa-Pro-Arg(Mtr)ψ($COCH_2$)Gly-OMe (3c). Boc-D,L-Dpa-Pro-Arg(Mtr)-OH (0.12 g, 0.15 mmol) was added to the anhydride of MMS (7 equiv, 1.05 mmol). Et_3N (0.33 mmol), DMAP (2.0 mg), and pyridine (0.13 mL) were added. The reaction mixture was stirred over a 45–50 °C water bath. After the reaction mixture was stirred for 1 h, 10 mL of 5% $NaHCO_3$ was added and the resultant mixture was stirred for an additional 30 min. The product was partitioned between ethyl acetate and water. The organic layer was washed with brine, dried (Na_2SO_4), and concentrated to dryness. The product was isolated by a flash silica gel column (2% methanol in $CHCl_3$ as eluent) to give **3c** (120 mg, 95%). ^{1}H NMR δ ($CDCl_3$): 7.35–7.21 (10 H, m, 2 C_6H_5), 6.52 (1 H, s, CH), 6.15 (1 H, m, NH), 4.98 (1 H, m, Dpa αCH), 4.54 (1 H, m, Arg CH), 4.35 (1 H, m, Dpa βCH), 4.17 (1 H, m, Pro CH), 3.81 (3 H, m, Mtr OCH_3), 3.67 (3 H, m, OCH_3), 2.72 (3 H, m, Mtr CH_3), 2.64 (3 H, m, Mtr CH_3), 2.12 (3 H, s, Mtr CH_3), 1.25 (9 H, m, t-C_4H_9), 3.45–1.10 (16 H, m, 8 CH_2). MS m/z (FAB): 877 ($M^{\cdot+}$).

Z-D-Dpa-Pro-Arg(Mtr)ψ($COCH_2$)Gly-OMe (3a) (134 mg, 98%). ^{1}H NMR δ ($CDCl_3$): 7.40–7.22 (15 H, m, 3 C_6H_5), 6.50 (1 H, d, CH), 5.98 (1 H, d, NH), 5.01 (1 H, m, Dpa αCH), 4.97 (2 H, m, OCH_2), 4.42 (1 H, m, Arg CH), 4.28 (1 H, m, Dpa βCH), 4.07 (1 H, m, Pro CH), 3.80 (3 H, d, Mtr OCH_3), 3.68 (3 H, d, OCH_3), 2.73 (3 H, m, Mtr CH_3), 2.64 (3 H, m, Mtr CH_3), 2.12 (3 H, d, Mtr CH_3), 3.45–1.10 (16 H, m, 8 CH_2).

Z-L-Dpa-Pro-Arg(Mtr)ψ($COCH_2$)Gly-OMe (3b) (127 mg, 92%). ^{1}H NMR δ ($CDCl_3$): 7.26–7.21 (15 H, m, 3 C_6H_5), 6.55 (1 H, m, NH), 6.51 (1 H, d, Mtr CH), 5.32 (1 H, m, NH), 5.02 (1 H, m, Dpa αCH), 4.97 (2 H, m, OCH_2), 4.46 (1 H, m, Arg CH), 4.35 (1 H, m, Dpa βCH), 4.08 (1 H, m, Pro CH), 3.81 (3 H, d, Mtr OCH_3), 3.68 (3 H, d, OCH_3), 2.70 (3 H, m, Mtr CH_3), 2.64 (3 H, m, Mtr CH_3), 2.12 (3 H, s, Mtr CH_3), 3.4–1.10 (16 H, m, 8 CH_2).

Boc-D,L-αNal-Pro-Arg(Mtr)ψ($COCH_2$)Gly-OMe (3d) (135 mg, 94%). Mp: 71–74 °C. ^{1}H NMR δ ($CDCl_3$): 8.15–7.33 (7 H, m, $C_{10}H_7$), 6.51 (1 H, s, CH), 6.35–6.03 (3 H, b, 3 NH), 5.25 (1 H, m, Nal CH), 4.45 (1 H, m, Arg CH), 4.14 (1 H, m, Pro CH), 3.79 (3 H, s, Mtr OCH_3), 3.67 (3 H, m, OCH_3), 3.62 (2 H, m, Nal CH_2), 2.68 (3 H, m, Mtr CH_3), 2.61 (3 H, m, Mtr CH_3), 2.11 (3 H, m, Mtr CH_3), 1.37 (9 H, d, t-Bu), 3.47–1.10 (16 H, m, 8 CH_2).

Boc-D,L-βNal-Pro-Arg(Mtr)ψ($COCH_2$)Gly-OMe (3e) (121 mg, 71%). R_f (A): 0.39. ^{1}H NMR δ ($CDCl_3$): 7.75–7.23 (7 H, m, $C_{10}H_7$), 6.55 (1 H, d, NH), 6.51 (1 H, d, Mtr CH), 6.23 (3 H, b, 3 NH), 4.74 (1 H, m, Nal CH), 4.41 (1 H, m, Arg CH), 4.07 (1 H, m, Pro CH), 3.81 (3 H, s, Mtr OCH_3), 3.65 (3 H, m, OCH_3), 3.46 (2 H, m, Nal CH_2), 2.68 (3 H, m, Mtr CH_3), 2.61 (3 H, m, Mtr CH_3), 2.11 (3 H, s, Mtr CH_3), 1.38 (9 H, m, t-C_4H_9), 3.50–1.10 (16 H, m, 8 CH_2).

Z-D-βNal-Pro-Arg(Mtr)ψ($COCH_2$)Gly-OMe (3f) (122 mg, 92%). ^{1}H NMR δ ($CDCl_3$): 7.85–7.42 (7 H, m, $C_{10}H_7$), 7.28 (5 H, s, C_6H_5), 6.49 (1 H, d, CH), 5.02 (2 H, m, OCH_2), 4.76 (1 H, m, Nal CH), 4.45 (1 H, m, Arg CH), 4.11 (1 H, m, Pro CH), 3.81 (3 H, d, Mtr OCH_3), 3.69 (3 H, d, OCH_3), 3.49 (2 H, m, Nal CH_2), 2.70 (3 H, m, Mtr CH_3), 2.64 (3 H, m, Mtr CH_3), 2.12 (3 H, d, Mtr CH_3), 3.48–1.10 (16 H, m, 8 CH_2).

Boc-D,L-Fgl-Pro-Arg(Mtr)ψ($COCH_2$)Gly-OMe (3g) (125 mg, 95%). ^{1}H NMR ($CDCl_3$): 7.80–7.20 (8 H, m, $C_{13}H_8$), 6.52 (1 H, d, NH), 6.48 (1 H, d, CH), 6.20 (3 H, b, 3 NH), 5.08 (1 H, m, Fgl αCH), 4.61 (1 H, m, Fgl βCH), 4.47 (1 H, m, Arg CH), 4.11 (1 H, m, Pro CH), 3.80 (3 H, m, Mtr OCH_3), 3.65 (3 H, m, OCH_3), 2.67 (3 H, m, Mtr CH_3), 2.61 (3 H, m, Mtr CH_3), 2.12 (3 H, m, Mtr CH_3), 1.22 (9 H, m, t-C_4H_9), 3.50–1.10 (16 H, m, 8 CH_2).

Preparation of Boc-D,L-Dpa-Arg(Mtr)ψ($COCH_2$)Gly-pip (4c). To a solution of **3c** (0.1 g, 0.11 mmol) in 10 mL of methanol cooled over an ice–water bath was added 1 N NaOH (2 equiv) with stirring. After stirring at room temperature for 2.5 h, it was brought to pH 7 with 1 N HCl. Methanol was removed in vacuo, and the aqueous solution was extracted with AcOEt and dried ($MgSO_4$). A crude solid was obtained after concentration to dryness.

To the solution of the crude solid and HOSu (13 mg, 0.11 mmol) in 20 mL of 1,2-dimethoxyethane was added DCC (23 mg, 0.11 mmol) with cooling. The mixture was stirred at room temperature for 20 h and then piperidine (19 mg, 0.22 mmol) was added with cooling. It was stirred at room temperature for 3 h. The solvent was evaporated in vacuo and the residue was purified through a flash silica gel column ($CHCl_3$ and then 2% methanol in $CHCl_3$ as eluent) to give **4c** (86 mg, 84%). Mp: 103–107 °C. ^{1}H NMR δ ($CDCl_3$): 7.34–7.21 (10 H, m, 2 C_6H_5), 6.51 (1 H, m, Mtr CH), 6.34 (1 H, m, NH), 6.18 (3 H, m, 3 NH), 5.99 (1 H, m, NH), 5.01 (1 H, m, Dpa αCH), 4.51 (1 H, Arg CH), 4.32 (1 H, m, Dpa βCH), 4.21 (1 H, m, Pro CH), 3.80 (3 H, m, Mtr OCH_3), 3.43 (4 H, m, pip CH_2), 2.71 (3 H, m, Mtr CH_3), 2.63 (3 H, m, Mtr CH_3), 2.12 (3 H, m, Mtr CH_3), 1.27 (9 H, m, t-C_4H_9), 3.28–1.10 (22 H, m, 11 CH_2). MS m/z (FAB): 930 ($M^{\cdot+}$).

Z-D-Dpa-Arg(Mtr)ψ($COCH_2$)Gly-pip (4a) (78 mg, 81%). R_f (A): 0.60. ^{1}H NMR δ ($CDCl_3$): 7.35–7.22 (15 H, m, 3 C_6H_5), 6.50 (1 H, d, CH), 6.30 (1 H, d, NH), 5.96 (3 H, b, 3 NH), 5.28 (1 H, d, NH), 4.97 (1 H, m, Dpa αCH), 4.85 (2 H, m, OCH_2), 4.54 (1 H, m, Arg CH), 4.37 (1 H, m, Dpa βCH), 4.28 (1 H, m, Pro CH), 3.81 (3 H, d, Mtr OCH_3), 3.36 (4 H, m, pip NCH_2), 2.69 (3 H, d, Mtr CH_3), 3.63 (3 H, d, Mtr CH_3), 2.11 (3 H, d, Mtr CH_3), 3.25–1.10 (22 H, m, 11 CH_2).

Z-L-Dpa-Pro-Arg(Mtr)ψ($COCH_2$)Gly-pip (4b) (80 mg, 75%). ^{1}H NMR δ ($CDCl_3$): 7.35–7.21 (15 H, m, 3 C_6H_5), 6.51 (1 H, d, CH), 5.25 (1 H, d, NH), 4.94 (1 H, m, Dpa αCH), 4.85 (1 H, m, OCH_2), 4.53 (1 H, m, Arg CH), 4.38 (1 H, m, Dpa βCH), 4.29 (1 H, m, Pro CH), 3.81 (3 H, d, Mtr OCH_3), 2.69 (3 H, d, Mtr CH_3), 3.62 (3 H, d, Mtr CH_3), 2.10 (CH_3, d, Mtr CH_3), 3.36–1.10 (26 H, m, 13 CH_2).

Boc-D,L-αNal-Pro-Arg(Mtr)ψ($COCH_2$)Gly-pip (4d) (107 mg, 88%). ^{1}H NMR δ ($CDCl_3$): 8.13–7.32 (7 H, m, $C_{10}H_7$), 6.51 (1 H, s, CH), 6.21 (3 H, m, 3 NH), 5.26 (1 H, m, Nal CH), 4.64 (1 H, m, Arg CH), 4.23 (1 H, m, Pro CH), 4.05 (3 H, m, Mtr OCH_3), 3.46 (2 H, m, Nal CH_2), 3.42 (4 H, m, pip NCH_2), 2.70 (3 H, m, Mtr CH_3), 2.62 (3 H, m, Mtr CH_3), 2.12 (3 H, m, Mtr CH_3), 1.27 (9 H, m, t-C_4H_9), 3.27–1.11 (22 H, m, 11 CH_2).

Boc-D,L-βNal-Pro-Arg(Mtr)ψ($COCH_2$)Gly-pip (4e) (90 mg, 62%). ^{1}H NMR δ ($CDCl_3$): 7.75–7.23 (7 H, m, $C_{10}H_7$), 6.51 (1 H, d, CH), 6.23 (3 H, b, 3 NH), 5.15 (1 H, m, Nal CH), 4.64 (1 H, m, Arg CH), 4.11 (1 H, m, Pro CH), 3.95 (3 H, m, Mtr OCH_3), 3.47 (2 H, m, Nal CH_2), 2.71 (3 H, m, Mtr CH_3), 2.64 (3 H, m, Mtr CH_3), 2.15 (3 H, m, Mtr CH_3), 1.26 (9 H, m, t-C_4H_9), 3.38–1.10 (26 H, m, 13 CH_2).

Z-D-βNal-Pro-Arg(Mtr)ψ($COCH_2$)Gly-pip (4f) (63 mg, 76%). ^{1}H NMR δ ($CDCl_3$): 7.85–7.41 (7 H, m, $C_{10}H_7$), 7.28 (5 H, s, C_6H_5), 6.51 (1 H, d, CH), 5.02 (2 H, m, OCH_2), 4.76 (1 H, m, Nal CH), 4.64 (1 H, m, Arg CH), 4.14 (1 H, m, Pro CH), 3.81 (3 H, d, Mtr OCH_3), 3.46 (2 H, m, Nal CH_2), 2.71 (3 H, m, Mtr CH_3), 2.65 (3 H, m, Mtr CH_3), 2.11 (3 H, m, Mtr CH_3), 3.45–1.11 (26 H, m, 13 CH_2).

Boc-D,L-Fgl-Pro-Arg(Mtr)ψ($COCH_2$)Gly-pip (4g) (89 mg, 87%). ^{1}H NMR δ ($CDCl_3$): 7.74–7.13 (8 H, m, $C_{13}H_8$), 6.50 (1 H, m, CH), 6.19 (3 H, m, 3 NH), 5.06 (1 H, m, Fgl αCH), 4.76 (1 H, m, Fgl βCH), 4.64 (1 H, m, Arg CH), 4.53 (1 H, m, Pro CH), 4.13 (3 H, m, Mtr OCH_3), 3.44 (4 H, m, pip CH_2), 2.75 (3 H, m, Mtr CH_3), 2.65 (3 H, m, Mtr CH_3), 2.12 (3 H, m, Mtr CH_3), 1.27 (9 H, m, t-C_4H_9), 3.25–1.10 (22 H, m, 11 CH_2).

Preparation of D,L-Dpa-Pro-Argψ($COCH_2$)Gly-pip (1c). To a mixture of 50 mg of compound **4c** and thioanisole (0.1 mL) cooled over an ice–water bath was added TFA (0.9 mL) with stirring. The reaction mixture was stirred at 0 °C for 30 min and at room temperature for 3 h. TFA was removed under reduced pressure. The residue was triturated with ether. The solid (**1c**)

(30 mg, 68%) was obtained by filtration, followed by washing with ether thoroughly, and dried. Mp: 121–126 °C. R_f (B): 0.46. HPLC (3): 14.8, 15.3 min. ^{1}H NMR δ ($CDCl_3$): 7.55–7.10 (10 H, m, 2 C_6H_5), 4.98 (1 H, m, Dpa αCH), 4.53 (1 H, m, Arg CH), 4.32 (1 H, m, Dpa βCH), 4.20 (1 H, m, Pro CH), 3.38–1.10 (26 H, m, 13 CH_2). MS m/z (FAB): 618 ($M^{\bullet+}$).

D-**Dpa-Pro-Argψ(COCH$_2$)Gly-pip (1a)** (30 mg, 75%). Mp: 146–151 °C. R_f (B): 0.41. HPLC (3): 14.73, 16.54 min. ^{1}H NMR δ ($CDCl_3$): 7.55–7.10 (10 H, m, 2 C_6H_5), 7.10–6.10 (4 H, m, guanidino H), 4.98 (1 H, m, Dpa αCH), 4.53 (1 H, m, Arg CH), 4.32 (1 H, m, Dpa βCH), 4.20 (1 H, m, Pro CH), 3.38–1.15 (26 H, m, 13 CH_2). MS m/z (FAB): 619 (M + H), 641 (M + Na). Anal. ($C_{34}H_{47}N_7O_4$·2TFA·4H_2O): C, H, N.

L-**Dpa-Pro-Argψ(COCH$_2$)Gly-pip (1b)** (30 mg, 43%). Mp: 136–140 °C. R_f (B): 0.41. ^{1}H NMR δ ($CDCl_3$): 7.43–7.10 (10 H, m, 2 C_6H_5), 4.99 (1 H, m, Dpa αCH), 4.56 (1 H, m, Arg CH), 4.35 (1 H, m, Dpa βCH), 4.20 (1 H, m, Pro CH), 3.40–1.15 (26 H, m, 13 CH_2). MS m/z (FAB): 619 (M + H). Anal. ($C_{34}H_{47}N_7O_4$·2CF_3CO_2H·3.5H_2O): C, H, N.

D,L-**αNal-Pro-Argψ(COCH$_2$)Gly-pip (1d)** (72 mg, 82%). Mp: 123–127 °C. HPLC (3): 13.33, 13.75 min. ^{1}H NMR δ ($CDCl_3$): 8.15–7.25 (7 H, m, $C_{10}H_7$), 5.26 (1 H, m, Nal CH), 4.64 (1 H, m, Arg αCH), 4.23 (1 H, m, Pro αCH), 3.46 (2 H, m, Nal CH_2), 3.42–1.10 (26 H, m, 13 CH_2). MS m/z (FAB): 592 ($M^{\bullet+}$). Anal. ($C_{32}H_{45}N_7O_4$·2TFA·3H_2O): C, H, N.

D,L-**βNal-Pro-Argψ(COCH$_2$)Gly-pip (1e)** (35 mg, 30%). MS m/z (FAB): 592 ($M^{\bullet+}$). Anal. ($C_{32}H_{45}N_7O_4$·2TFA·3.5H_2O): C, H, N.

D-**βNal-Pro-Argψ(COCH$_2$)Gly-pip (1f)** (28 mg, 68%). HPLC (3): 11.6, 12.4 min. ^{1}H NMR δ ($CDCl_3$): 7.70–7.21 (7 H, m, $C_{10}H_7$), 4.78 (1 H, m, Nal CH), 4.46 (1 H, m, Arg CH), 4.18 (1 H, m, Pro CH), 3.47 (2 H, m, Nal CH_2), 3.40–1.10 (26 H, m, 13 CH_2). MS m/z (FAB): 593 (M + H). Anal. ($C_{32}H_{45}N_7O_4$·2TFA·4H_2O): C, H, N.

D,L-**Fgl-Pro-Argψ(COCH$_2$)Gly-pip (1g)** (59 mg, 81%). R_f (B): 0.46. ^{1}H NMR δ ($CDCl_3$): 7.77–7.15 (8 H, m, $C_{13}H_8$), 5.04 (1 H, m, Fgl αCH), 4.76 (1 H, m, Arg CH), 4.64 (1 H, m, Fgl βCH), 4.53 (1 H, m, Pro CH), 3.45–1.05 (26 H, m, 13 CH_2). MS m/z (FAB): 6.17 ($M^{\bullet+}$). Anal. ($C_{34}H_{45}N_7O_4$·2TFA·3.5H_2O): C, H. N.

Enzymatic Assay.[15] Kinetic studies were made in 0.1 M sodium phosphate buffer containing 0.2 M NaCl, 0.5% polyethylene glycol 6000, and 0.02% sodium azide at pH 7.5. The assays were performed in the presence or absence of inhibitor at a concentration sufficient to give approximately 50% inhibition at a single concentration of substrate S-2238 (10 μM). The assay was carried out with a range of substrate concentrations at and around the K_m for substrate/thrombin (20–5 μM). The final concentration of human α-thrombin[16] used was 0.19 nM, and the assays were carried out at 37 °C. The inhibition constant (K_i) was calculated from a Lineweaver–Burk plot.

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3′,3′-Difluoro-3′-deoxythymidine: Comparison of Anti-HIV Activity to 3′-Fluoro-3′-deoxythymidine

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3′,3′-Difluoro-3′-deoxythymidine (3) has been synthesized in four steps from thymidine, and characterized by ^{1}H NMR and NOE experiments. The J_{HF} coupling constants support a conformation in solution that is predominantly 2′-endo (*S*). Although conformationally and sterically nucleoside 3 may resemble other thymidine analogs which are active against HIV-1, 3 is virtually inactive.

One of the most active nucleoside analogs against HIV-1 is 3′-fluoro-3′-deoxythymidine (1).[3,4] This nucleoside is a more potent inhibitor of HIV-1 replication in vitro than 3′-azidothymidine (2) (Table I). We previously reported that the analog with an additional 3′-fluoro substituent, 3′,3′-difluoro-3′-deoxythymidine (3), is virtually inactive against HIV-1.[5] On the basis of calculations using MINDO/3, a semiempirical, all-valence-electron molecular orbital method, we postulated that the 3′-fluoro, located on the same face of the furanose as the thymine, strongly influenced the conformation about the glycosidic bond and led to a substantial preference for the N (3′-endo, 2′-exo) conformation of the deoxyribose. On the basis of NMR experiments reported here, that prediction is not born out.

The synthesis of 3′,3′-difluoro-3′-deoxythymidine is outlined in Figure 1. Although the synthesis was previously reported, the experimental details, the structure determination, and the data on antiviral activity have not been published previously.[6,7] The first two steps, tritylation of thymidine and the oxidation of 5′-*O*-trityl-thymidine to yield 3′-ketothymidine (5) have been previously described in detail.[8] Fluorination was accom-

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