Development of Methodology for the Synthesis of Stereochemically Pure PheΨ[CH_2N]Pro Linkages in HIV Protease Inhibitors

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One of the strategies currently being pursued for the design of potential HIV protease inhibitors involves the replacement of the cleaved amide bond in a minimum peptide substrate with an aminomethylene unit. A commonly used method for the synthesis of these compounds involves a reductive alkylation of an amine with an aldehyde in the presence of sodium cyanoborohydride under acidic conditions. Accordingly, BOC-phenylalaninal (4) was reacted with the peptide-resin ProIleSer(OBzl)OResin (5) in the presence of acetic acid and sodium cyanoborohydride. The resulting product was found to consist of a mixture of diastereomers, which may result from the fact that the proline residue, which contains a secondary amine, reacts with the aldehyde to form an enamine 9 with loss of chirality at the modified Phe residue. Subsequent reduction of the iminium ion 10 would then result in production of the observed two diastereomers. In order to circumvent this problem, BOCPheProOBzl (12b) was synthesized and the central amide bond was reduced selectively with diborane. Hydrogenolysis of the benzyl protecting group gave BOCPheΨ[CH_2N]Pro (14a), which was coupled manually to the peptide resin IleSer(OBzl)OResin to give BOCPheΨ[CH_2N]ProIleSer(OBzl)OResin (6). Subsequent addition of amino acid residues to 6 and cleavage from the resin gave a series of stereochemically defined potential HIV protease inhibitors as single diastereomers. The most potent of these substances was ThrLeuAsnPheΨ[CH_2N]ProIleSer (1), which displayed an IC_{50} of 1.1 μg/mL (1.4 μM) when tested for inhibition of HIV-1 protease. However, the epimer of 1 having the opposite configuration at the reduced Phe residue was inactive. A minimum length of seven amino acid residues appears to be necessary for effective recognition of the inhibitor by the enzyme. Further increase in chain length did not result in greater inhibitory potency.

The replication competent retroviruses, including HIV, encode a protease which cleaves the *gag* and *pol* precursor polyproteins into the functional proteins of the mature viruses.[1-3] In vitro mutagenesis that produces protease-defective virus results in the formation of uninfective, immature forms of the virus.[4-6] Therefore, inhibition of the protease constitutes a rational strategy for the development of potential anti-AIDS agents. During the course of a program designed to synthesize inhibitors of HIV protease, we wished to prepare a series of pseudopeptides in which the cleaved PhePro amide bonds of substrate peptides were replaced by noncleavable aminomethylene (CH_2N) units.

The initial targets chosen for synthesis were the peptide derivatives 1, 2, and 3.[7-9] The sequence of amino acid residues in these peptides corresponds to the cleavage site between the protease and the reverse transcriptase in the HIV-1 *gag-pol* polyprotein.[2] A method in general use for the replacement of amide bonds in peptides with the CH_2N bond involves reductive alkylation of an amine with an aldehyde.[10] Accordingly, BOC-L-phenylalaninal 4 was

ThrLeuAsnPheΨ[CH_2N]ProIleSer
1

LeuAsnPheΨ[CH_2N]ProIle
2

CysThrLeuAsnPheΨ[CH_2N]ProIleSerProIle
3

added to the peptide-resin 5 in *N*,*N*-dimethylformamide (DMF) containing 1% acetic acid, followed by sodium cyanoborohydride to give the peptide-resin 6. The aldehyde 4 was prepared by the lithium aluminum hydride reduction of N^{α}-(*tert*-butoxycarbonyl)-L-phenylalanine *N*-methoxy-*N*-methylamide (8), which reportedly gives the product in high optical purity.[11] The peptide resin 6 was deprotected and the ThrLeuAsn segment was added automatically by solid-phase peptide synthesis (SPPS). The peptide 1 was cleaved from the resin using anhydrous HF (Scheme I). Using this methodology, the peptides 2 and 3 were also prepared.

Scheme I

$$\underset{4}{\text{BOCPheCHO}} + \underset{5}{\text{ProIleSer(OBzl)OResin}} \xrightarrow[1\%\ \text{AcOH}]{\text{NaBH}_3\text{CN, DMF}} \underset{6}{\text{BOCPheΨ[CH}_2\text{N]ProIleSer(OBzl)OResin}} \xrightarrow[3.\ \text{HF}]{1.\ \text{TFA}\ \ 2.\ \text{SPPS}} \underset{1}{\text{TheLeuAsnPheΨ[CH}_2\text{N]ProIleSer}}$$

The analytical HPLC traces produced by the crude peptides 1-3 showed that each had two major peaks (Figure 1). The compounds producing these major peaks were isolated by semipreparative HPLC. FABMS analysis indicated that in each case the products were mixtures of

(1) Yoshinaka, T.; Katoh, I.; Copeland, T.; Oroszlan, S. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 1618.
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(3) Yoshinaka, Y.; Katoh, O.; Copeland, T.; Smythers, G. W.; Oroszlan, S. *J. Virol.* **1986**, *57*, 826.
(4) Katoh, I.; Yoshinaka, Y.; Rein, A.; Shibuya, M.; Okada, T.; Oroszlan, S. *Virology* **1985**, *145*, 280.
(5) Crawford, S.; Goff, S. P. *J. Virol.* **1985**, *53*, 899.
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(8) Billich, S.; Knoop, M.-T.; Hansen, J.; Strop, P.; Sedlacek, J.; Mertz, R.; Moelling, K. *J. Biol. Chem.* **1988**, *263*, 17095.
(9) Darke, P. L.; et al. *Biochem. Biophys. Res. Commun.* **1988**, *156*, 297. Darke, P. L.; Leu, C.-T.; Davis, L. J.; Heimbach, J. C.; Diehl, R. E.; Hill, W. S.; Dixon, R. A. F.; Sigal, I. S. *J. Biol. Chem.* **1989**, *264*, 2307.
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(11) Fehrentz, J.-A.; Castro, B. *Synthesis* **1983**, 676.

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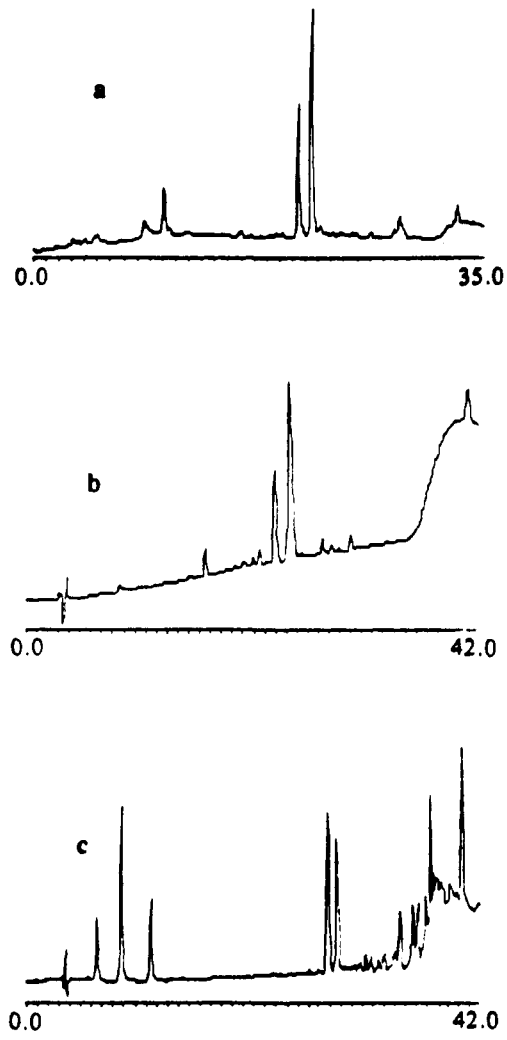


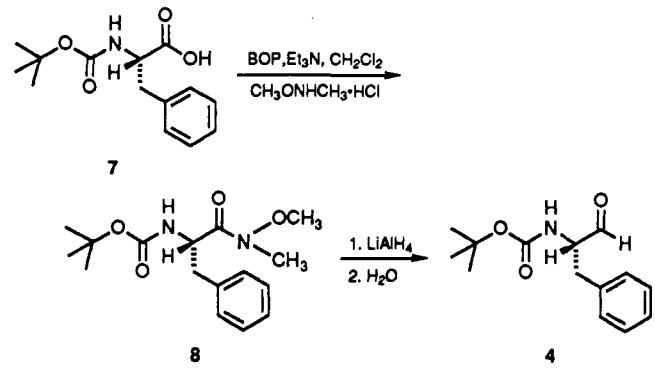
Figure 1. Analytical HPLC traces of the crude peptides obtained by reductive alkylation method: (a) ThrLeuAsnPheΨ[CH_2N]-ProIleSer; (b) LeuAsnPheΨ[CH_2N]ProIle; (c) CysThrLeuAsnPheΨ[CH_2N]ProIleSerProIle.

diastereomers. Therefore, an attempt was made to pinpoint the causes of this undesired production of diastereomers. One possibility is loss of optical purity during the $LiAlH_4$ reduction of the *N*-methoxy-*N*-methylamide **8** to the aldehyde **4**. Recently Coy and co-workers have also examined the optical purity of BOC-L-phenylalaninal **4** and BOC-D-phenylalaninal and found that substantial racemization could occur during the aldehyde synthesis if a large excess of $LiAlH_4$ was used.[12] Only when a small excess of $LiAlH_4$ was used at 0 °C was racemization reduced to undetectable levels, as indicated by the production of only one diastereomer when the aldehyde was used in the solid phase synthesis of L-PheΨ[CH_2NH]-L-Leu-NH_2.[12] In our hands, a small-scale reduction of *N*-methoxy-*N*-methylamide **8** with 0.8 equiv of $LiAlH_4$ at −50 °C for 20 min gave aldehyde having 88% enantiomeric excess, as indicated by the optical rotation of the product. Higher optical purities were not achieved despite considerable experimentation with the reaction conditions. When the scale was increased 18-fold, the optical purity dropped to 68% enantiomeric excess (see the Experimental Section).

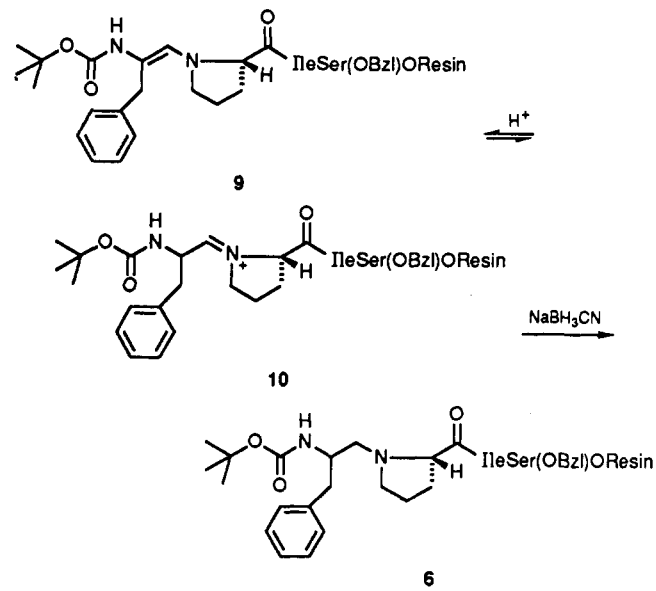
During the course of our work a fresh supply of peptide **1** was needed and prepared using the same strategy. Although the ratio of BOC-L-phenylalaninal to BOC-D-phenylalaninal in the starting material was 84:16, respectively, HPLC analysis of the crude peptide product showed that the ratio of diastereomers present was 3:2. It became obvious that racemization during aldehyde synthesis could not be the sole reason for the production of diastereomers. Racemization of BOC-L-phenylalaninal itself in the reaction mixture used for reductive alkylation and epimerization during HF cleavage from the resin can both be ruled out on the basis of previously published work.[12] A possible explanation is that proline, being a secondary amine, forms an enamine intermediate **9** which results in loss of chirality in the phenylalanine residue (Scheme III). Subsequent reduction of the iminium ion **10** would then result in a diastereomeric mixture of peptide-resins, which after further peptide synthesis and cleavage from the resin would give the observed diastereomeric mixtures of products. Although the diastereomers could be separated by preparative HPLC, it was not possible to assign the configurations of the reduced phenylalanine residues in the two products. The production of diastereomers during reductive alkylations of proline residues in peptides has not previously been noted in the literature.

An alternative strategy which leads to a single, stereochemically defined diastereomer has to avoid enamine formation. This can be achieved if a stereochemically pure reduced dipeptide derivative **14a** is synthesized and incorporated into the peptide chain.[13] Although it might be expected that the selective diborane reduction of the amide carbonyl group in dipeptides should proceed with

Scheme II



Scheme III



(12) Coy, D. H.; Hacart, S. J.; Sasaki, Y. *Tetrahedron* **1988**, *44*, 835.

(13) Heimbach, J. C.; Garsky, V. M.; Michelson, S. R.; Dixon, R. A. F.; Sigal, I. S.; Darke, P. L. *Biochem. Biophys. Res. Commun.* **1989**, *164*, 955.

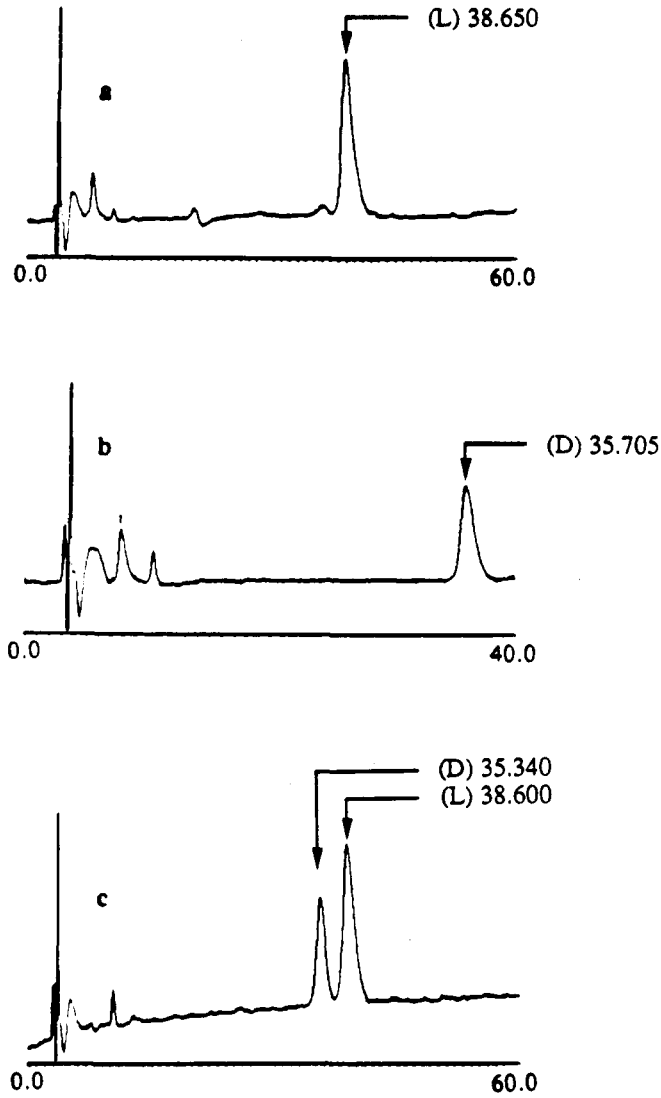


Figure 2. Analytical HPLC traces of diborane reduction products: (a) BOC-L-PheΨ[CH_2N]ProOMe; (b) BOC-D-PheΨ[CH_2N]-ProOMe; (C) co-injection. Buffers: A = 0.1% TFA in H_2O; B = 0.1% TFA in 60% CH_3CN–H_2O. Isocratic at 35% B. Detector: 220 nm. The numbers next to each peak indicate retention times in minutes.

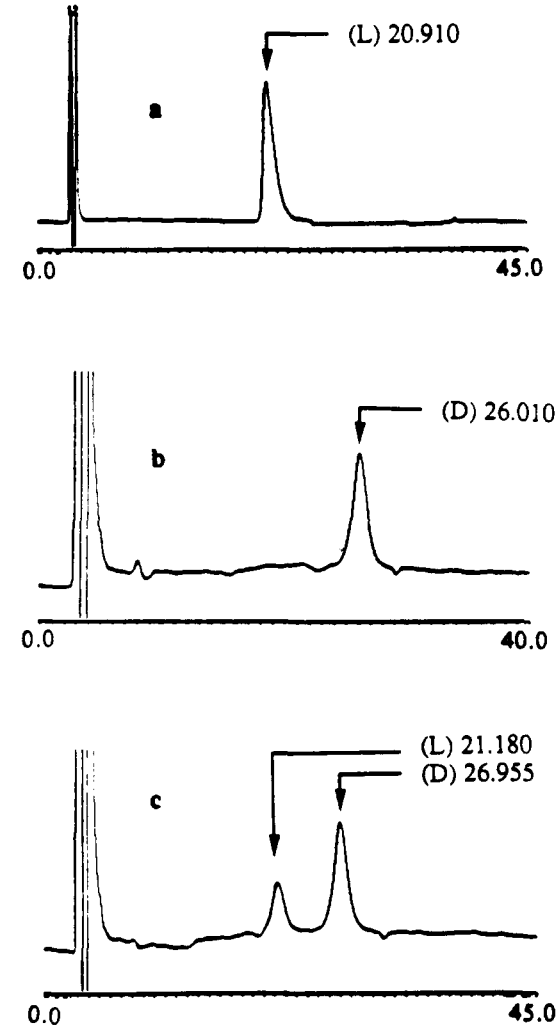
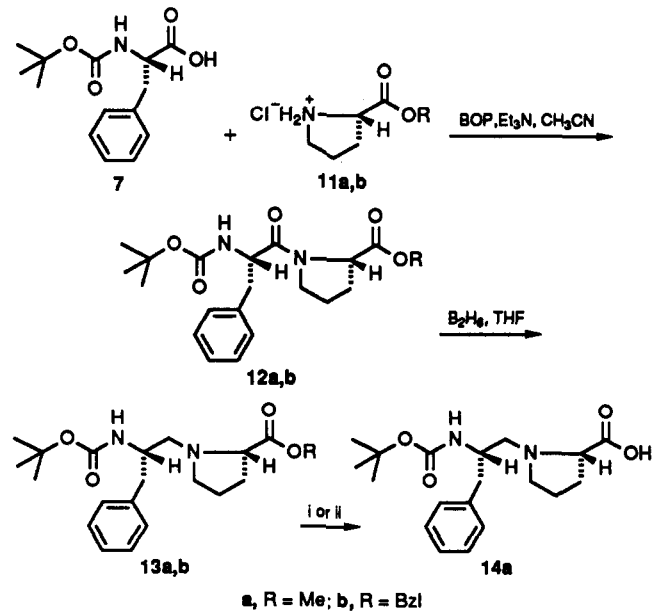


Figure 3. Analytical HPLC traces of saponification products: (a) BOC-L-PheΨ[CH_2N]Pro; (b) BOC-D-PheΨ[CH_2N]Pro; (c) co-injection. Buffers: A = 0.1% TFA in H_2O; B = 0.1% TFA in 60% CH_3CN–H_2O. Isocratic at 35% B. Detector: 220 nm. The numbers next to each peak indicate retention times in min.

retention of stereochemistry at the two asymmetric centers, the previous work reported in this area does not prove this point.[15b] Proline methyl ester hydrochloride (**11a**) was reacted with BOC-L-phenylalanine (**7**) and (benzotriazolyloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP) to give the dipeptide **12a**,[14] which was reduced with diborane in tetrahydrofuran to the dipeptide **13a**[15a] (Scheme IV). In a parallel sequence, the dipeptide BOC-D-PheΨ[CH_2N]ProOMe was prepared from BOC-D-phenylalanine and proline methyl ester hydrochloride. HPLC analysis of both BOC-L-PheΨ-[CH_2N]ProOMe **13a** and BOC-D-PheΨ[CH_2N]ProOMe confirmed that the diborane reduction did not result in epimerization at either of the two asymmetric centers (Figure 2). The methyl ester **13a** and BOC-D-PheΨ-[CH_2N]ProOMe were saponified with 1 N NaOH to give dipeptide derivatives BOC-L-PheΨ[CH_2N]ProOH (**14a**) and BOC-D-PheΨ[CH_2N]ProOH without any detectable

Scheme IV[a]



a, R = Me; b, R = Bzl

[a] (i) 1 N NaOH, MeOH; (ii) H_2, Pd/C, MeOH.

(14) Castro, B.; Dormoy, J. R.; Dourtoglou, B.; Evin, G.; Selve, C.; Zigler, J.-C. *Synthesis* **1976**, 751.

(15) (a) Brown, H. C.; Heim, P. *J. Org. Chem.* **1973**, *38*, 912. (b) Roeske, R. W.; Weitl, F. L.; Prassad, K. U.; Thompson, R. M. *J. Org. Chem.* **1976**, *41*, 1260.

epimerization as evidenced by HPLC analysis (Figure 3).

Although this procedure was free of epimerization, the chemical yield was unacceptably low (37%) due to the complicated workup. The use of a benzyl ester protecting group and hydrogenolysis for deprotection gave the dipeptide derivative **14a** in high yield (94%).

When the dipeptide derivative **14a** was manually coupled to the peptide-resin **15** in the presence of DCC and HOBt, the peptide-resin **6** was obtained. The peptide-resin **6** was deprotected, the ThrLeuAsn segment was added automatically on the peptide synthesizer, and the resulting peptide **1** was cleaved from the resin using HF (Scheme V). HPLC analysis of the crude peptide showed that only one diastereomer was present.

Scheme V

BOCPheΨ[CH_2N]Pro (**14a**) + IleSer(OBzl)OResin (**15**) —DCC, HOBt, DMF→ BOCPheΨ[CH_2N]ProIleSer(OBzl)OResin (**6**) —1. TFA, 2. SPPS, 3. HF→ PheΨ[CH_2N]ProIleSer (**16**) and AsnPheΨ[CH_2N]ProIleSer (**17**) and ThrLeuAsnPheΨ[CH_2N]ProIleSer (**1**)

Since one of the objectives of this work was to determine the minimum chain length required for inhibition of the enzyme, a series of stereochemically pure peptides was prepared using a similar methodology (Schemes V, VI, and VII).

Scheme VI

BOCPheΨ[CH_2N]Pro (**14a**) + IleOResin (**18**) —DCC, HOBt, DMF→ BOCPheΨ[CH_2N]ProIleOResin (**19**) —1. TFA, 2. SPPS, 3. HF→ AsnPheΨ[CH_2N]ProIle (**20**) and LeuPheΨ[CH_2N]ProIle (**21**) and LeuAsnPheΨ[CH_2N]ProIle (**2**)

Scheme VII

BOCPheΨ[CH_2N]Pro (**14a**) + IleSer(OBzl)ProIleOResin (**22**) —DCC, HOBt, DMF→ BOCPheΨ[CH_2N]ProIleSer(OBzl)ProIleOResin (**23**) —1. TFA, 2. SPPS, 3. HF→ CysThrLeuAsnPheΨ[CH_2N]ProIleSerProIle (**3**)

As mentioned earlier, the peptides **1**, **2**, and **3**, in which the reduced amide linkages were introduced to the resin-bound segment by reductive alkylation of the nitrogen in proline with BOC-phenylalaninal **4** (Scheme I), were contaminated with a significant amount of diastereomeric material as evidenced by HPLC and FABMS analysis. This prevented a certain configurational assignment of the isolated diastereomers. Since the stereochemically pure peptides, LeuAsnPheΨ[CH_2N]ProIle, **2**, ThrLeuAsnPheΨ[CH_2N]ProIleSer, **1**, and CysThrLeuAsnPheΨ[CH_2N]ProIleSerProIle, **3**, were synthesized by the other method, it became possible to assign the configuration of each diastereomer by HPLC analysis. It was found that the diastereomers of **1** and **3** with the shorter retention times, and the diastereomer of **2** with the longer retention time had the L configuration at the reduced phenylalanine residue. The latter case comes in clear contrast to the observation by Goodman and Schiller[16] that "mixed chirality peptides are more hydrophobic than peptides composed of amino acids of a single chirality".

Table I. Inhibition of HIV-1 Protease by Noncleavable Peptide Substrate Analogues Containing Aminomethylene Linkages[a]

inhibitor	IC_{50} (μM)
PheΨ[CH_2N]ProOBz	–
PheΨ[CH_2N]ProOH	–
PheΨ[CH_2N]ProIleSer	>550
AsnPheΨ[CH_2N]ProIle	–
LeuPheΨ[CH_2N]ProIle	–
LeuAsnPheΨ[CH_2N]ProIle	931
AsnPheΨ[CH_2N]ProIleSer	439
ThrLeuAsn-D-PheΨ[CH_2N]ProIleSer	–
ThrLeuAsnPheΨ[CH_2N]ProIleSer	1.4
CysThrLeuAsnPheΨ[CH_2N]ProIleSerProIle	3.4
CysThrLeuAsn-D-PheΨ[CH_2N]ProIleSerProIle	37.3

[a] The assay was performed according to the published procedure.[19] The substrate utilized was SP211, VSQNYPIVQ-NH_2 at a concentration of 5 μg/12.5 μL. The enzyme was HIV-1 protease expressed from *E. coli*.[20] The buffer was 100 mM MES, pH 6, 3 M NaCl, 10% glycerol, 2 mM EDTA, and 2 mM DDT. Each of the reagents (12.5 μL) was mixed, and incubation was allowed to proceed for 0 and 2 h. The reaction was quenched with guanidine chloride (60 μL, 8 M) and TFA (10 μL, 20% v/v). The products were analyzed and quantitated by HPLC on a NOVAPAK C_{18} column (4.9 s 150 mm), eluting with a gradient of 0–20% acetonitrile in 15 min.

The dipeptides **24** and **25** were obtained by treating the BOC protected peptides, **13b** and **14a**, with trifluoroacetic acid in dry CH_2Cl_2. HPLC analysis of both **24** and **25** showed that they were stereochemically pure (Scheme VIII).

Scheme VIII

BOCPheΨ[CH_2N]ProOBz (**13b**) —TFA, CH_2Cl_2→ PheΨ[CH_2N]ProOBz (**24**)

BOCPheΨ[CH_2N]Pro (**14a**) —TFA, CH_2Cl_2→ PheΨ[CH_2N]Pro (**25**)

For the biological tests, the assay used was that developed by Copeland and Oroszlan in which HIV-1 protease, substrate, and inhibitor were incubated together for the required period of time.[17] All the peptides that were shorter than seven amino acid residues in length were either inactive or very poor inhibitors. On the other hand, the heptapeptide ThrLeuAsnPheΨ[CH_2N]ProIleSer completely inhibited the enzyme at a concentration of 7.5 μg/mL, and it had a calculated IC_{50} of 1.1 μg/mL (1.4 μM). Its diastereomer having a D-Phe residue, however, was inactive as an inhibitor of HIV-1 protease (Table I). For comparison, the IC_{50} previously reported for CysThrLeuAsnPheΨ[CH_2N]ProIleSerProIle against HIV-1 protease is 125 μM.[8]

From the results presented in Table I, it is clear that there is a relationship between the number of amino acid residues surrounding the PheΨ[CH_2N]Pro site and HIV-1 protease inhibitory activity. Although some inhibition was seen with the two reduced pentapeptides LeuAsnPheΨ-

(16) Richman, S. J.; Goodman, M.; Nguyen, Thi, M.-D.; Schiller, P. W. *Int. J. Peptide Protein Res.* **1985**, *25*, 648.
(17) Copeland, T. D.; Oroszlan, S. *Gene Anal. Tech.* **1988**, *5*, 109.

[CH_2N]ProIle and AsnPheΨ[CH_2N]ProIleSer, their potencies were low when compared with that of the reduced heptapeptide ThrLeuAsnPheΨ[CH_2N]ProIleSer. A further increase in chain length to the decapeptide analogue CysThrLeuAsnPheΨ[CH_2N]ProIleSerProIle did not result in an increase in inhibitory potency. However, when the inactive, reduced D-phenylalanine-containing heptapeptide analogue ThrLeuAsn-D-PheΨ[CH_2N]ProIleSer was extended to the decapeptide analogue CysThrLeuAsn-D-PheΨ[CH_2N]ProIleSerProIle, some protease inhibitory activity resulted (IC_{50} 37.3 μM). These results on the minimal length needed for HIV-1 protease inhibitory activity are in harmony with several other studies that have indicated that a minimal length of six or seven amino acid residues in peptide substrates are needed for efficient peptide cleavage.[8,9,18,19]

Experimental Section

Microanalyses were performed by the Purdue Microanalytical Laboratory. Analytical thin-layer chromatography was done on Baker-flex silica gel 1B2-F plastic coated sheets and Merck silica 60 F_{254} glass coated plates. Flash column chromatography was performed using 230–400-mesh silica gel. Analytical reverse-phase HPLC was performed on a 4.6 × 250 mm, 10 μm, C-18, Vydac 218TP column. Semipreparative HPLC was accomplished on a 10 × 350 mm, 12 μm, C-18, Dynamax 300A column.

Distilled, deionized water, Fisher Scientific HPLC grade acetonitrile, and Chemical Dynamics Corporation's sequalog grade trifluoroacetic acid were used in all HPLC work. THF was dried by distillation from potassium benzophenone ketyl. *N,N*-Dimethylformamide was distilled from calcium hydride and stored over molecular sieves. Triethylamine was distilled from calcium hydride and stored over potassium hydroxide pellets. Organic solutions were dried over $MgSO_4$.

***N*-(*tert*-Butoxycarbonyl)-L-phenylalaninal (4).** Lithium aluminum hydride (0.276 g, 7.27 mmol) was added to a stirred cold (−50 °C) solution of *N*-(*tert*-butoxycarbonyl)-L-phenylalanine *N*-methoxy-*N*-methylamide[11] (1.79 g, 5.82 mmol) in ether (29 mL). The reaction mixture was stirred for 20 min at −50 °C and then hydrolyzed with a solution of potassium hydrogen sulfate (1.39 g, 10.2 mmol) in water (30 mL) at −50 °C. The mixture was extracted with ether (100 mL followed by 3 × 50 mL). The combined organic extract was washed with sodium hydrogen carbonate solution (3 × 30 mL) and saturated sodium chloride solution (3 × 20 mL), dried, and concentrated under reduced pressure to afford the aldehyde 4 (1.68 g) as a solid: mp 86–87 °C (lit.[11] mp 86 °C); $[\alpha]_D$ = +27.5 (*c* 1.0, CH_2Cl_2) [lit.[11] $[\alpha]_D$ = +40.4 (*c* 1.0, CH_2Cl_2)]; ^1H NMR (200 MHz, $CDCl_3$) δ 1.42 (9 H, s), 3.10 (d, 2 H, *J* = 6.4 Hz), 4.38 (1 H, m), 5.22 (1 H, d, *J* = 6.5 Hz), 7.20–7.35 (5 H, m), 9.59 (1 H, s).

General Procedure for the Reductive Coupling of Aldehyde 4 to Resins. A solution of the aldehyde 4 (2.5 equiv) in DMF containing 1% acetic acid (7 mL/0.5 mmol of resin) was added to the resin with agitation. Solid sodium cyanoborohydride (2.5 equiv) was added in one portion, and the mixture was agitated for 3 h. The resin was filtered off, washed with DMF (2 × 20 mL), methanol (2 × 30 mL), and methylene chloride (2 × 30 mL), and air-dried.

BOCPheProOMe (12a). Triethylamine (0.556 mL, 4 mmol) was added to a solution of Boc-L-phenylalanine (530 mg, 2 mmol), L-proline methyl ester hydrochloride (331 mg, 2 mmol), and BOP (885 mg, 2 mmol) in acetonitrile (30 mL). The mixture was stirred at room temperature for 1 h, saturated sodium chloride solution (100 mL) was added, and the mixture was extracted three times with ethyl acetate (3 × 100 mL). The organic phase was washed successively with 2 N hydrochloric acid (50 mL), 10% sodium hydrogen carbonate (50 mL), and water (50 mL) and dried. The solvent was removed under reduced pressure to give the crude peptide **12a** as an oil. The peptide was purified by flash chromatography on silica gel (80 g, 4.5 × 10 cm, 1:5 ethyl acetate–hexane) to yield the purified peptide **12a** (735 mg, 97.7%) as an oil: IR (neat) 3360, 2860, 1710, 1500, 1450, 1360, 1160 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 1.43 (9 H, s), 1.91–2.18 (4 H, m), 2.91 (1 H, dd, *J* = 13.7, 6.7 Hz), 3.09 (1 H, dd, *J* = 14, 7 Hz), 3.20 (1 H, m), 3.59 (1 H, m), 3.75 (3 H, s), 4.50 (1 H, dd, *J* = 8, 4.2 Hz), 4.65 (1 H, q, *J* = 7 Hz), 5.27 (1 H, d, *J* = 7.1 Hz), 7.29 (5 H, s); CIMS *m/e* (relative intensity) 277 (16.3), 321 (40.7), 377 (100.0). Anal. Calcd for $C_{20}H_{28}N_2O_5$: C, 63.81; H, 7.50; N, 7.44. Found: C, 63.64; H, 7.82; N, 7.32.

BOC-D-PheProOMe (12c). This peptide was obtained in a manner identical with the preparation of **12a**, as an oil: IR (neat) 3380, 2880, 1740, 1700, 1640, 1490, 1430, 1160 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 1.44 (9 H, s), 1.75–2.27 (4 H, m), 2.64 (1 H, dd, *J* = 7.9, 5.3 Hz), 2.91 (1 H, m), 3.06 (1 H, dd, *J* = 13.1, 5.5 Hz), 3.49 (1 H, m), 3.71 (3 H, s), 4.30 (1 H, dd, *J* = 7.3, 4.3 Hz), 4.63 (1 H, dd, *J* = 8.8, 5.6 Hz), 5.38 (1 H, d, *J* = 8.2 Hz), 7.24 (5 H, s); CIMS *m/e* (relative intensity) 277 (6.4), 315 (5.7), 321 (9.5), 377 (100.0). Anal. Calcd for $C_{20}H_{28}N_2O_5$: C, 63.81; H, 7.50; N, 7.44. Found: C, 63.46; H, 7.81; N, 7.83.

BOCPheProOBz (12b). Triethylamine (6.30 mL, 45.2 mmol) was added to a solution of BOC-L-phenylalanine (6.00 g, 22.6 mmol), L-proline benzyl ester hydrochloride (5.47 g, 22.6 mmol), and BOP (10.0 g, 22.6 mmol) in acetonitrile (45 mL). The mixture was stirred at room temperature for 1.5 h, saturated sodium chloride solution (100 mL) was added, and the mixture was extracted three times with ethyl acetate (3 × 150 mL). The organic phase was washed successively with 3 N hydrochloride acid (3 × 150 mL), 5% sodium hydroxide (3 × 150 mL), and water (1 × 150 mL) and dried. The solvent was removed under reduced pressure to give the crude peptide **12b** as a solid. The crude peptide was purified by recrystallization from ether to yield the purified peptide **12b** (8.91 g, 19.7 mmol, 87.1%) as a white crystalline solid: mp 101–103 °C; ^1H NMR (500 MHz, $CDCl_3$) δ 1.37 (9 H, s), 1.89–1.97 (3 H, m), 2.15–2.19 (1 H, m), 2.85 (1 H, dd, *J* = 13.7, 7 Hz), 3.04 (1 H, dd, *J* = 13.8, 6.7 Hz), 3.20 (1 H, dt, *J* = 9.8, 6.2 Hz), 3.58–3.64 (1 H, m), 4.56 (1 H, dd, *J* = 8.9, 4.5 Hz), 4.64 (1 H, ddd, *J* = 8.9, 7.0, 6.7 Hz), 5.17 (1 H, d, *J* = 12.4 Hz), 5.21 (1 H, d, *J* = 12.4 Hz), 5.26 (1 H, d, *J* = 8.9 Hz), 7.21–7.28 (5 H, m), 7.32–7.37 (5 H, m). Anal. Calcd for $C_{26}H_{32}N_2O_5$: C, 69.01; H, 7.13; N, 6.19. Found: C, 68.93; H, 6.87; N, 6.34.

BOCPheΨ[CH_2N]ProOMe (13a). Peptide **12a** (267 mg, 0.710 mmol) was added to a solution of borane (1 M in THF; 1.42 mL, 1.42 mmol) at 0 °C under nitrogen over a period of 5 min. After the addition was complete, the resulting mixture was stirred at room temperature for 24 h. After the reaction flask was cooled to 0 °C, saturated potassium hydrogen sulfate (20 mL) was added slowly. The THF was removed in vacuo using a rotary evaporator. Sodium hydroxide was added to saturate the aqueous phase, and the latter was extracted three times with a total of 150 mL of ethyl acetate. The organic solution was dried and concentrated under reduced pressure to afford the reduced peptide **13a** as an oil. The reduced peptide was purified by flash chromatography on silica gel (30 g, 2.5 × 12 cm, 1:5 ethyl acetate–hexane) to yield the purified peptide **13a** (130 mg, 0.358 mmol, 50.4%) as an oil: IR (neat) 3320, 3000, 1750, 1710, 1650, 1440, 1140 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 1.41 (9 H, s), 1.71–2.09 (4 H, m), 2.50 (1 H, m), 2.54 (1 H, dd, *J* = 12.6, 6.7 Hz), 2.69 (1 H, dd, *J* = 12.6, 6.7 Hz), 2.92 (2 H, m), 3.25 (2 H, m), 3.71 (3 H, s), 3.83 (1 H, dd, *J* = 12, 6.7 Hz), 4.77 (1 H, bs), 7.19–7.31 (5 H, m); CIMS *m/e* (relative intensity) 89 (100.0), 142 (14.8), 307 (12.3), 363 (53.3). Anal. Calcd for $C_{20}H_{30}N_2O_4$: C, 66.27; H, 8.34; N, 7.73. Found: C, 66.46; H, 8.64; N, 7.55.

BOC-D-PheΨ[CH_2N]ProOMe (13c). This peptide was prepared in a manner identical with the preparation of **13a** as an oil (29.6%): IR (neat) 3400, 2990, 1710, 1490, 1160, cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 1.4 (9 H, s), 1.7–2.0 (4 H, m), 2.0 (1 H, m), 2.3 (1 H, dd, *J* = 12.5, 7 Hz), 2.4 (1 H, dd, *J* = 12.8, 5.6 Hz), 2.6

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(2 H, m), 3.1 (2 H, m), 3.25 (1 H, dd, J = 9.6, 6.4 Hz), 3.7 (3 H, s), 5.1 (1 H, bs), 7.2–7.3 (5 H, m); CIMS m/e (relative intensity) 89 (39.1), 142 (14.5), 307 (12.3), 363 (100.0). Anal. Calcd for $C_{20}H_{30}N_2O_4$: C, 66.27; H, 8.34; N, 7.73. Found: C, 66.38; H, 8.69; N, 7.85.

BOCPheΨ[CH$_2$N]ProOBz (13b). Peptide **12b** (3.53 g, 7.81 mmol) was added to a solution of borane (1 M in THF; 15.6 mL, 15.6 mmol) in THF (52 mL) at 0 °C under nitrogen over a period of 5 min. After the addition was complete, the resulting mixture was stirred at room temperature for 24 h. The reaction flask was cooled to 0 °C, and saturated potassium hydrogen sulfate (50 mL) was added slowly. The THF was evaporated in vacuo using a rotary evaporator. Sodium carbonate (50 mL) was added to saturate the aqueous phase, and the latter was extracted three times with a total of 450 mL of ethyl acetate. The organic solution was dried and concentrated under reduced pressure to afford the reduced peptide **13b** as a solid. This peptide was partially purified by flash chromatography on silica gel (96 g, 4.5 × 12 cm, 1:8 ethyl acetate–hexane) and further purified by recrystallization from ethyl acetate–hexane (1:5) to give the purified peptide **13b** (1.81 g, 4.13 mmol, 52.9%) as a white crystalline solid: mp 78–79 °C; IR (KBr) 3360, 2990, 1710, 1495, 1450, 1360, 1160 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.39 (9 H, s), 1.77–1.85 (1 H, m), 1.88–1.97 (2 H, m), 2.03–2.12 (1 H, m), 2.51–2.56 (2 H, m), 2.67 (1 H, dd, J = 12.5, 7.0 Hz), 2.93 (1 H, dd, J = 13.9, 6.2 Hz), 3.17 (1 H, dt, J = 12.3, 3.9 Hz), 3.30 (1 H, dd, J = 8.5, 4.8 Hz), 3.80–3.90 (1 H, m), 4.70–4.80 (1 H, m), 5.13 (1 H, d, J = 12.3 Hz), 5.17 (1 H, d, J = 12.3 Hz), 7.17–7.28 (5 H, m), 7.33–7.37 (5 H, m); CIMS m/e (relative intensity) 218 (11.9), 303 (6.7), 439 (100.0). Anal. Calcd for $C_{27}H_{34}N_2O_4$: C, 71.21; H, 7.81; N, 6.39. Found: C, 71.39; H, 7.94; N, 6.13.

BOCPheΨ[CH$_2$N]ProOH (14a) from 13a. To a solution of the reduced peptide **13a** (423 mg, 1.17 mmol) in methanol (9 mL) was added 1 N NaOH (2.34 mL, 2.34 mmol), and the mixture was stirred for 6 h at room temperature. The methanol was evaporated and the aqueous residue was washed with ethyl acetate (5 mL) and then acidified with solid citric acid to pH 4–5 with cooling. The desired product precipitated out during the neutralization. The precipitate was collected, washed with cold water and dried in vacuo. The crude peptide was purified by recrystallization from methanol–water (1:10) to yield pure **14** (171 mg, 0.434 mmol, 37.1%) as a white crystalline solid: mp 156–159 °C; IR (neat) 3300, 2990, 1700, 1390, 1360, 1170 cm^{-1}; ^{1}H NMR (200 MHz, DMSO-d_6) δ 1.3 (9 H, s), 1.7–2.1 (4 H, m), 2.6–3.0 (3 H, m), 3.2–3.4 (4 H, m), 3.7 (1 H, m), 6.8 (1 H, d, J = 8.5 Hz), 7.2–7.3 (5 H, m); low resolution FABMS MH$^+$ m/e 349.

BOCPheΨ[CH$_2$N]ProOH (14a) from 13b. A solution of the dipeptide **13b** (1.59 g, 3.62 mmol) in methanol (159 mL) containing 10% Pd on charcoal (159 mg) was stirred under a hydrogen atmosphere for 1 h. The reaction mixture was filtered through Celite, and the methanol was removed in vacuo. The residue was purified by recrystallization from methanol–ethyl acetate (1:5) to yield the purified peptide (1.19 g, 3.41 mmol, 94.3%) as a white crystalline solid: mp 156–159 °C; ^{1}H NMR (500 MHz, CD_3OD) δ 1.35 (9 H, s), 1.90–2.01 (1 H, m), 2.04–2.15 (2 H, m), 2.31–2.39 (1 H, m), 2.74 (1 H, dd, J = 13.6, 9.1 Hz), 2.95 (1 H, dd, J = 13.7, 5.2 Hz), 3.10–3.19 (2 H, m), 3.40 (1 H, dd, J = 13.1, 4.8 Hz), 3.70–3.76 (1 H, m), 3.82–3.88 (1 H, m), 4.07 (1 H, ddd, J = 13.7, 8.7, 2.0 Hz), 7.21–7.30 (5 H, m). Anal. Calcd for $C_{20}H_{28}N_2O_4$: C, 65.49; H, 8.10; N, 8.04. Found: C, 65.09; H, 7.98; N, 7.92.

BOC-D-PheΨ[CH$_2$N]ProOH-CF$_3$COOH (14b). This peptide was prepared in a manner identical with the preparation of **14a** as an oil. The crude product was purified on a Vydac 218 TP, C-18, 15–20 μm, 2.25 × 25 cm column. A gradient of 18% CH_3CN–0.1% TFA to 27% CH_3CN–0.1% TFA in 30 min was used at a flow rate of 10 mL/min. The desired fraction was lyophilized to give the pure peptide (30%): IR (neat) 3354, 2970, 1694, 1496, 1454, 1170 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.39 (9 H, s), 1.83 (1 H, m), 2.06 (3 H, m), 2.74 (1 H, dd, J = 13.6, 8.2 Hz), 2.80 (2 H, m), 3.01 (1 H, dd, J = 13.6, 6.4 Hz), 3.46 (1 H, m), 3.86 (3 H, m), 4.16 (1 H, m), 5.80 (1 H, d, J = 8.2 Hz); low-resolution FABMS MH$^+$ m/e 349. Anal. Calcd for $C_{21}H_{29}N_2O_6F_3$: C, 54.54; H, 6.32; N, 6.06. Found: C, 54.44; H, 6.67; N, 5.85.

General Procedure for the Coupling of the Dipeptides 14a and 14b to Resins. Under an atmosphere of nitrogen, a mixture containing the peptide **14a** or **14b** (435 mg, 1.25 mmol), resin (0.5 mmol), DCC (258 mg, 1.25 mmol), and HOBt·H_2O (191 mg, 1.25 mmol) was stirred in dry DMF (10 mL) for 24 h at room temperature. The resulting resin was filtered off, washed with DMF (3 × 30 mL), methanol (3 × 50 mL), and methylene chloride (3 × 20 mL), and air-dried. The yield of the coupling was determined by a quantitative ninhydrin test.

Purification of ThrLeuAsnPheΨ[CH$_2$N]ProIleSer (1). The crude peptide (110 mg) was purified on a Dynamax 300 A, C-18, 12 μm, 10 × 350 mm column. A gradient of 9% CH_3CN–0.1% TFA to 30% CH_3CN–0.1% TFA in 30 min was used at a flow rate of 3 mL/min. The desired fractions were lyophilized, dissolved in water, and filtered through Amberlite IRA-400 (OAc) ion exchange resin. The eluents were lyophilized to give the pure peptide **1** (66.1 mg). High resolution FABMS calcd MH$^+$ m/e 777.4511 found 777.4496.

Purification of ThrLeuAsn-D-PheΨ[CH$_2$N]ProIleSer. The crude peptide (19.8 mg) was purified on a Dynamax 300 A, C-18, 12 μm, 10 × 350 mm column. A gradient of 9% CH_3CN–aqueous triethylammonium phosphate (pH 6.5) to 30% CH_3CN–aqueous triethylammonium phosphate (pH 6.5) in 20 min and an isocratic of 30% CH_3CN–aqueous triethylammonium phosphate (pH 6.5) in 8 min were used at a flow rate of 3 mL/min. The desired fractions were lyophilized, and the triethylammonium phosphate was removed on a Dynamax 300 A, C-18, 12 μm, 10 × 350 mm column. A gradient of 9% CH_3CN–0.1% TFA to 12% CH_3CN–0.1% TFA in 5 min and 12% CH_3CN–0.1% TFA to 36% CH_3CN–0.1% TFA in 10 min and an isocratic of 36% CH_3CN–0.1% TFA in 5 min were used at a flow rate of 3 mL/min. The desired fractions were lyophilized, dissolved in water, and filtered through Amberlite IRA-400 (OAc) ion exchange resin. The eluents were lyophilized to give the pure peptide (7.3 mg): high-resolution FABMS calcd MH$^+$ m/e 777.4511 found 777.4541.

Purification of LeuAsnPheΨ[CH$_2$N]ProIle (2). The crude peptide (20.6 mg) was purified on a Dynamax 300 A, C-18, 12 μm, 10 × 350 mm column. A gradient of 15% CH_3CN-0.1% TFA to 24% CH_3CN–0.1% TFA in 30 min and an isocratic of 24% CH_3CN–0.1% TFA in 10 min were used at a flow of 3 mL/min. The desired fractions were lyophilized, dissolved in water, and filtered through Amberlite IRA-400 (OAc) ion exchange resin. The eluents were lyophilized to give the pure peptide **2** (12.5 mg): high-resolution FABMS m/e calcd MH$^+$ 589.3714, found 589.3721.

Purification of PheΨ[CH$_2$N]ProIleSer (16). The crude peptide **16** (17.5 mg) was purified on a Dynamax 300 A, C-18 12 μm, 10 × 350 mm column. A gradient of 9% CH_3CN–0.1% TFA to 15% CH_3CN–0.1% TFA in 10 min and an isocratic of 15% CH_3CN–0.1% TFA in 15 min were used at a flow rate of 3 mL/min. The desired fractions were lyophilized, dissolved in water, and filtered through a column of Amberlite IRA-400 (OAc) ion exchange resin. The eluents were lyophilized to give the pure peptide **16** (15.4 mg): high-resolution FABMS m/e calcd MH$^+$ 449.2764 found 449.2748.

Purification of AsnPheΨ[CH$_2$N]ProIleSer (17). The crude peptide (39.5 mg) was purified on a Dynamax 300 A, C-18, 12 μm, 10 × 350 mm column. An isocratic of 11% CH_3CN–0.1% TFA in 40 min was used at a flow rate of 3 mL/min. The desired fractions were lyophilized, dissolved in water, and filtered through Amberlite IRA-400 (OAc) ion exchange resin. The eluents were lyophilized to give the pure peptide **17** (7.9 mg): high-resolution FABMS m/e calcd MH$^+$ 563.3193 found 563.3172.

Purification of AsnPheΨ[CH$_2$N]ProIle (20). The crude peptide (8.1 mg) was purified on a Dynamax 300 A, C-18, 12 μm, 10 × 350 mm column. A gradient of 12% CH_3CN–0.1% TFA to 24% CH_3CN–0.1% TFA in 30 min and an isocratic of 24% CH_3CN–0.1% TFA in 10 min were used at a flow rate of 3 mL/min. The desired fractions were lyophilized, dissolved in water, and filtered through Amberlite IRA-400 (OAc) ion exchange resin. The eluents were lyophilized to give the pure peptide **20** (3.6 mg): high-resolution FABMS m/e calcd MH$^+$ 476.2873 found 476.2878.

Purification of LeuPheΨ[CH$_2$N]ProIle (21). The crude peptide (40.3 mg) was purified on a Dynamax 300 A, C-18, 12 μm, 10 × 350 mm column. A gradient of 9% CH_3CN–0.1% TFA to 33% CH_3CN–0.1% TFA in 22.5 min was used at a flow rate of 3 mL/min. The desired fractions were lyophilized, dissolved in water, and filtered through Amberlite IRA-400 (OAc) ion exchange

resin. The eluents were lyophilized to give the pure peptide **21** (14.5 mg): high-resolution FABMS m/e calcd MH^+ 475.3284 found 475.3280.

PheΨ[CH_2N]ProOBz (24). Trifluoroacetic acid (6.0 mL) was added to an ice-cold solution of *N*-*t*-BOC-PheΨ[CH_2N]ProOBz (100 mg, 0.228 mmol) in dry CH_2Cl_2 (6.0 mL). After the addition was complete, the ice bath was removed and the mixture was stirred at room temperature for 30 min. The volatiles were removed on a rotary evaporator, and the product was washed with hexane (2 × 10 mL). The solid product was dried under reduced pressure to afford the peptide **24** (86.2 mg), which was 95% pure as determined by analytical HPLC: mp 139–140 °C; ^1H NMR (500 MHz, CD_3OD) δ 1.72–1.81 (1 H, m), 1.82–1.96 (2 H, m), 2.11–2.17 (1 H, m), 2.57 (2 H, d, J = 5.0 Hz), 2.73 (1 H, m), 2.75 (1 H, dd, J = 13.5, 8.9 Hz), 2.92 (1 H, m), 2.94 (1 H, dd, J = 13.3, 5.3 Hz), 3.22 (1 H, dd, J = 9.6, 8.5 Hz), 3.73 (1 H, ddd, J = 11.1, 9.0, 5.5 Hz), 4.8 (1 H, d, J = 12.0 Hz), 4.9 (1 H, d, J = 12.0 Hz), 7.22–7.25 (5 H, m), 7.30–7.33 (5 H, m); high-resolution FABMS m/e calcd MH^+ 399.2072, found 399.2035.

PheΨ[CH_2N]ProOH (25). Trifluoroacetic acid (3.1 mL) was added to an ice-cold solution of *N*-*t*-BOCPheΨ[CH_2N]ProOH (40.0 mg, 0.115 mmol) in dry CH_2Cl_2 (3.1 mL). After the addition was complete, the ice bath was removed and the mixture was stirred at room temperature for 30 min. The volatiles were removed on a rotary evaporator, and the product was dissolved in water (5 mL). The aqueous solution was washed with ether (4 × 10 mL) and lyophilized. The crude dipeptide **25** was purified on a Dynamax 300 A, C-18, 12 μm, 10 × 350 mm column. A gradient of 0% CH_3CN–0.1% TFA to 18% CH_3CN–0.1% TFA in 30 min was used at a flow rate of 3 mL/min. The desired fractions were lyophilized, dissolved in water, and filtered through Amberlite IRA-400 (OAc) ion exchange resin. The eluents were lyophilized to give the pure peptide **25** (12.5 mg, 0.05 mmol): mp 189–191 °C; ^1H NMR (500 MHz, CD_3OD) δ 1.70–1.82 (2 H, m), 1.89–1.96 (1 H, m), 2.16–2.24 (1 H, m), 2.53–2.58 (1 H, m), 2.63–2.67 (1 H, dd, J = 13.4, 4.1 Hz), 2.77–2.82 (1 H, dd, J = 13.3, 11.0 Hz), 2.84–2.90 (1 H, m), 2.90 (2 H, dd, J = 9.8, 5.7 Hz), 3.19–3.15 (1 H, ddd, J = 8.7, 6.4, 2.7 Hz), 3.25–3.35 (1 H, m), 7.23–7.35 (5 H m); high-resolution FABMS m/e calcd MH^+ 249.1603 found 249.1599.

Purification of CysThrLeuAsnPheΨ[CH_2N]ProIleSerProIle (3). The crude peptide (5 mg) was purified on a Dynamax 300A, C-18, 12 μm, 10 × 350 mm column. A gradient of 9% CH_3CN–0.1% TFA to 36% CH_3CN–0.1% TFA in 30 min was used at a flow rate of 3 mL/min. The desired fraction was lyophilized, dissolved in water, and filtered through Amberlite IRA-400 (OAc^-) ion exchange resin. The eluents were lyophilized to give the pure peptide **3** (0.25 mg): low-resolution FABMS m/e 1090 (MH^+).

Purification of CysThrLeuAsnPheΨ[CH_2N]ProIleSerProIle (3) and CysThrLeuAsn-D-PheΨ[CH_2N]ProIleSerProIle. The crude peptide (15 mg, from reductive alkylation method) was purified on a Dynamax 300A, C-18, 12 μm, 10 × 350 mm column. Isocratic elution with 24% CH_3CN–0.1% TFA was used at a flow rate of 3 mL/min. The desired fractions were lyophilized, dissolved in water, and filtered through Amberlite IRA-400 (OAc^-) ion exchange resin. The eluents were lyophilized to give the pure L-peptide (5.2 mg) and its D-Phe diastereomer (4.4 mg): low-resolution FABMS calcd MH^+ m/e 1090.58, found 1090.55 for both diastereomers.

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Supplementary Material Available: HPLC analyses of the purified peptides (11 pages). Ordering information is given on any current masthead page.

Synthesis of Four Diastereomeric L-2-(Carboxycyclopropyl)glycines. Conformationally Constrained L-Glutamate Analogues

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To determine what conformations of L-glutamate (L-Glu) activate that compound's different receptors in the mammalian central nervous system, four diastereomeric L-2-(carboxycyclopropyl)glycines, **1–4**, which are conformationally constrained analogues of the extended and folded conformers of L-Glu, were synthesized and subjected to neutrophysiological assay. Compounds **1–4** were efficiently synthesized from chiral amino acids. Cyclopropanation of the (2*S*)-2-amino-3-butenol derivative **5b** gave intermediates for the synthesis of all four diastereomers. Stereoselective cyclopropanation of both the α,β-unsaturated γ-lactam **16** and the δ-lactone **19** gave precursors of (2*S*,1′*S*,2′*R*)-**3** and (2*S*,1′*R*,2′*S*)-**4**, respectively. Neurophysiological assays of **1–4** performed with the newborn rat spinal cord demonstrated that the compounds induced a variety of depolarizing effects. The results of the assays strongly suggested that the *N*-methyl-D-aspartic acid (NMDA) receptor is activated by the folded conformer of L-Glu and that the extended conformer of L-Glu activates the metabotropic L-Glu receptor. The four analogous D-2-(carboxycyclopropyl)glycines (D-**1**–D-**4**), which were synthesized from (2*R*)-**5b**, proved to be NMDA agonists.

Introduction

The neurobiological effects that L-glutamate (L-Glu) induces in the mammalian central nervous system (CNS) are well-documented. L-Glu acts chiefly as an excitatory neurotransmitter, and its excitotoxic effect is closely related to ischemic neuron damage.[1] Also, L-Glu is believed to play a role in the construction of memory and in early

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(1) For reviews, see: (a) Monaghan, D. T.; Bridges, R. J.; Cotman, C. W. *Ann. Rev. Pharmacol. Toxicol.* **1989**, *29*, 365. (b) Shinozaki, H. *Progress in Neuropharmacol.* **1988**, *30*, 399.

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