Ketomethylene and (Cyanomethylene)amino Pseudopeptide Analogues of the C-Terminal Hexapeptide of Neurotensin

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A series of pseudopeptide analogues of the C-terminal hexapeptide of neurotensin (NT$_{8-13}$), namely [Tyr$^{11}\Psi$[COCH$_2$]Phe^{12}]-, [Ile$^{12}\Psi$[COCH$_2$]Phe^{13}]-, and [Tyr$^{11}\Psi$[CH(CN)NH]Ile^{12}]NT$_{8-13}$ with different stereochemistries, has been synthesized and evaluated for its potency in displacing labeled NT from rat cortex membranes. Ketomethylene pseudohexapeptides were prepared from the corresponding Boc-protected ketomethylene dipeptide derivatives, previously formed, using different solid phase synthesis (SPS) conditions, while (cyanomethylene)amino analogues were directly prepared by SPS using Fmoc strategy. H-Arg-Arg-Pro-TyrΨ[COCH$_2$]-Phe-Leu-OH was nearly as potent as NT$_{8-13}$ and [Phe^{12}]NT$_{8-13}$ in binding to the receptor. Comparison of the affinities for the pseudohexapeptides, here reported, with those of the Ψ-[CH$_2$NH] analogues indicates the importance of the CO group in the amide or surrogate linkage at 11−12 and 12−13 positions in the receptor binding process.
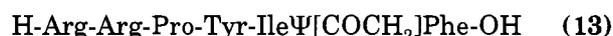
Introduction

Neurotensin (NT) is a biologically active peptide largely distributed in the central nervous system and some regions of the digestive tract in various mammals, including man.[1-3] It has been shown that this peptide produces hypotension, increases vascular permeability, possesses antinociceptive properties, and elicits antipsychotic-like effects in animal tests.[3,4] The C-terminal hexapeptide of NT, H-Arg-Arg-Pro-Tyr-Ile-Leu-OH (NT$_{8-13}$), contains all the necessary information to trigger the biological response of NT.[5,6]

In an attempt to provide resistance to peptidases, a systematic replacement of each peptide bond in NT$_{8-13}$ with the reduced Ψ[CH$_2$NH] isostere was performed.[7-9] Except for the [Arg$^8\Psi$[CH$_2$NH]Arg9]NT$_{8-13}$ derivative, these replacements resulted in a decrease in affinity for NT receptors, particularly marked in pseudohexapeptides incorporating the reduced bond between the 11−12 and 12−13 residues. Similar results were found when the Ile^{12}−Leu^{13} peptide bond was replaced with the retro-amide isostere.[10]

Now, in order to further investigate the functional role of the Tyr^{11}−Ile^{12} and Ile^{12}−Leu^{13} amide bonds in NT$_{8-13}$, we have selected the ketomethylene [COCH$_2$] and (cyanomethylene)amino [CH(CN)NH] groups as appropriate surrogates for this purpose. Thus, similarly to the introduction of the Ψ[CH$_2$NH] bond into peptides, the popular COCH$_2$ group, widely used for the preparation of metabolically stable neuropeptide analogues[11] and enzyme inhibitors,[12,13] increases the conformational freedom.[14] In contrast, the hydrogen bond properties of both isosteres are opposite (donor or acceptor). Concerning the recently reported CH(CN)NH group, semiempirical quantum mechanic calculations have indicated that, due to its electronic properties, it could be a better mimic of the amide bond than the CH$_2$NH

substitute.[15-17] Additionally, in this new peptide bond surrogate, the cyano group keeps H-bonding acceptor properties while the new asymmetric center could impart higher backbone rigidity than the reduced peptide bond.

In the present article, we report the preparation of a series of NT$_{8-13}$ analogues, **12−14**, incorporating Ψ-[COCH$_2$] and Ψ[CH(CN)NH] surrogates at 11−12 and 12−13 positions and their ability to inhibit the binding of ^{3}H-labeled NT to rat cortex membranes. The results of the binding assays are compared with those of the corresponding hexapeptides and reduced peptide bond analogues.

H-Arg-Arg-Pro-TyrΨ[COCH$_2$]Phe-Leu-OH   (**12**)

H-Arg-Arg-Pro-Tyr-IleΨ[COCH$_2$]Phe-OH   (**13**)

H-Arg-Arg-Pro-TyrΨ[CH(CN)NH]Ile-Leu-OH   (**14**)

Results and Discussion

Chemistry. Considering that in our previous binding assays the substitution of Ile^{12} or Leu^{13} by a Phe residue did not modify the activity of NT$_{8-13}$, and in order to facilitate the synthetic pathway, the ketomethylene pseudodipeptides to be incorporated into the NT$_{8-13}$ sequence bear a Phe side chain at the C-terminus. Protected ketomethylene derivatives Boc-Tyr(2,6-di-Cl-Bzl)-Ψ[COCH$_2$](R,S)Phe-OH (**9**) and Boc-IleΨ[COCH$_2$](R,S)Phe-OH (**10**) were prepared following a similar method to that previously reported for the preparation of these peptide bond surrogates (Scheme 1).[18,19] Thus, the γ-keto diesters **3** and **4**, prepared by reaction of the corresponding chloromethyl ketones **1** and **2** with the monosodium salt of dibenzyl malonate, were alkylated with benzyl bromide, using sodium hydride as base, to provide the 2-disubstituted derivatives **5** and **6**, respectively. Hydrogenolysis of compounds **5** and **6**, followed by decarboxylation of the resulting malonic acid derivatives **7** and **8**, afforded

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Scheme 1

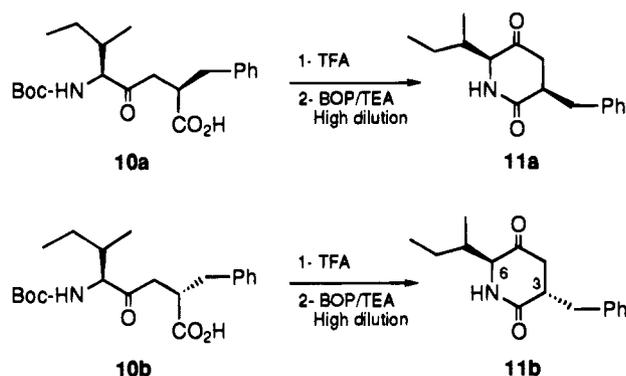**1, 3, 5, 7, 9** : R = CH$_2$Ph[4-O-(2',6'-di-Cl-Bzl)]

2, 4, 6, 8, 10: R = CH(CH$_3$)CH$_2$CH$_3$
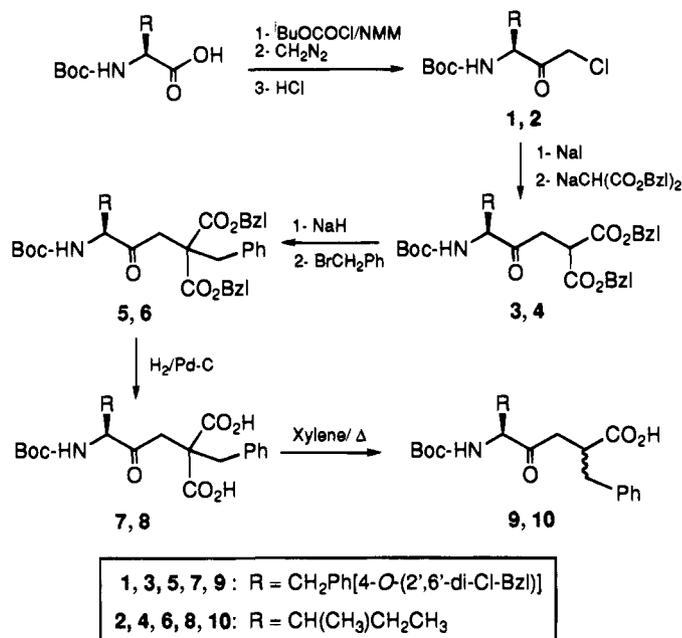
Scheme 2

pseudodipeptides **9** and **10** as 1:1 mixtures of diastereoisomers. In the case of ketomethylene dipeptide analogue **10**, which, after a tedious flash chromatography could be separated into the two diastereoisomers, the determination of the absolute configuration at the new asymmetric center was performed by a ^{1}H NMR study of the corresponding 2,5-diketopiperidines **11a,b** (Scheme 2).[20] Thus, for lactam **11b**, obtained by BOP-mediated cyclization of the lower R_f pseudodipeptide derivative **10b**, a shielding effect was observed for the H-6 proton when compared to the same proton in the 2,5-diketopiperidine **11a**, indicating that this proton is *cis* to the 3-benzyl moiety. As the absolute configuration at C-6 is *S*, due to the starting l-Ile, the absolute configuration at C-3 is *R* in compounds **10a** and **11a** and *S* in their epimers **10b** and **11b**.

H-Arg-Arg-Pro-TyrΨ[COCH$_2$](*R*)Phe-Leu-OH (**12a**) and H-Arg-Arg-Pro-TyrΨ[COCH$_2$](*S*)Phe-Leu-OH (**12b**) were prepared by coupling of Boc-Tyr(2,6-di-Cl-Bzl)Ψ-[COCH$_2$](*R,S*)Phe-OH (**9**) to H-Leu-PAM resin followed by solid phase synthesis (SPS) and HF cleavage. As expected, HPLC analysis of the resulting crude product showed the presence of two stereoisomers that were separated by semipreparative HPLC. Comparison of the ^{1}H NMR spectra of these two isomeric pseudohexapeptides revealed that the Leu γ-CH proton in the

Table 1. Epimerization at Ile C$^\alpha$ of Ketomethylene Pseudopeptides **10a** and **13a** under Basic Conditions

conditions	compound (%)	
	10a	**13a**
formation of Cs salt, 18 h in DMF at 50 °C	>45^a	–
DMAP (0.1 equiv) DCM, 18 h	<1	<1
10% DIEA, DMF, 30 min	2	1
20% piperidine, DMF, 2.5 h	4	20

^{a} Similar results were found with isomer **10b**.

diastereoisomer **12b** (0.92 ppm) is more shielded than in the analogue **12a** (1.54 ppm). This significant shielding, similar to that previously reported for various LD and DL diastereomeric dipeptides or dipeptide fragments with one aromatic amino acid and the other aliphatic,[21,22] allowed us to assign the absolute configuration at the Phe residue in **12a,b** as *R* (L) and *S* (D), respectively.

The C-terminal ketomethylene pseudohexapeptides H-Arg-Arg-Pro-Tyr-IleΨ[COCH$_2$](*R*)Phe-OH (**13a**) and H-Arg-Arg-Pro-Tyr-IleΨ[COCH$_2$](*S*)Phe-OH (**13b**) were initially prepared by esterification of chloromethylene resin with the Cs^{2+} salt of the diastereoisomeric mixture of the pseudodipeptides **10** followed by SPS and HF cleavage. Unexpectedly, this methodology also led to the two additional isomers **13c,d** resulting from epimerization of **13a,b**, respectively, at the Ile C$^\alpha$. In fact, model experiments performed with both isomers **10a,b** indicated that significant epimerization took place during treatment of the Cs salt of these pseudodipeptides with DMF at 50 °C (Table 1), normal conditions used for the attachment of an amino acid or peptidic fragment to chloromethyl poly(styrene) resin. A similar cesium salt-mediated epimerization was reported for Ψ[CH$_2$S] pseudodipeptide analogues. [23] However, this epimerization was completely avoided when the ketomethylene dipeptide derivative **10** was anchored to hydroxymethyl resin using DCC/DMAP. In this case, repetition of the SPS starting from a 9:1 mixture of **10a,b** only provided pseudohexapeptides **13a,b** in the same ratio, indicating that no epimerization took place. Based on the fact that **13c** comes from epimerization of **13a** at the Ile C$^\alpha$ position and on the Tyr^{11} shielding effect on the Ile γ-CH$_2$ and γ-CH$_3$ protons of derivatives **13c,d** with respect to those of **13a,b** (Table 4), the configurations at the C-terminal dipeptide fragment were established as LL, LD, DL, and DD for **13a**−**d**, respectively.

The facility of ketomethylene pseudopeptides to undergo epimerization at the α-CH position adjacent to the COCH$_2$ moiety during basic treatments, commonly used in SPS, was investigated with both the dipeptide derivative **10a** and the pseudohexapeptide **13a**. From these experiments, it can be concluded that DIEA treatment, corresponding to 15 cycles of Boc SPS, does not cause significant epimerization, whereas in the piperidine treatment, corresponding to 15 cycles of Fmoc SPS, appreciable epimerization occurs (Table 1). Therefore, Boc methodology and esterification of hydroxymethyl resin with DCC/DMAP are the strategies of choice for the general preparation of ketomethylene pseudopeptide analogues in solid phase.

(Cyanomethylene)amino analogues of NT$_{8-13}$, H-Arg-Arg-Pro-TyrΨ[(*R*)CH(CN)NH]Ile-Leu-OH (**14a**) and H-Arg-Arg-Pro-TyrΨ[(*S*)CH(CN)NH]Ile-Leu-OH (**14b**), were prepared by SPS using Fmoc strategy followed by TFA cleavage.[24] The introduction of the Ψ[CH(CN)NH]

Scheme 3
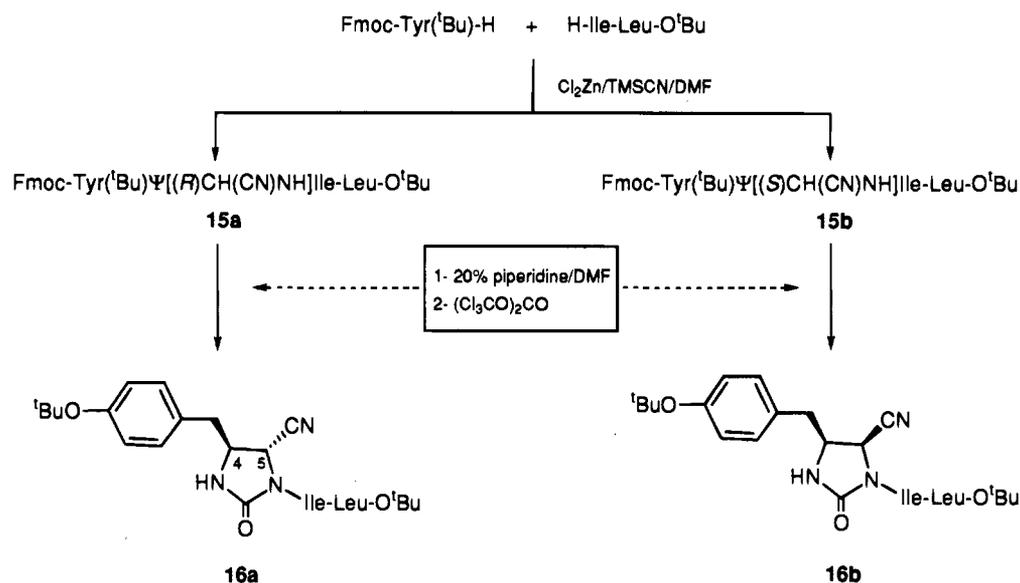
Table 2. Receptor Binding of $\Psi[COCH_2]$- and $\Psi[CH(CN)NH]NT_{8-13}$ Analogues

		absolute configuration				
compound	isomer	pos 12	pos 13	CH(CN)	IC$_{50}$ (nM)^a	rel potency
H-Arg-Arg-Pro-TyrΨ[COCH$_2$]Phe-Leu-OH	**12a**	R	S		15	66.6
	12b	S	S		1200	0.8
H-Arg-Arg-Pro-Tyr-IleΨ[COCH$_2$]Phe-OH	**13a**	S	R		120	8.3
	13b	S	S		1200	0.8
	13c	R	R		1500	0.6
	13d	R	S		900	1.1
H-Arg-Arg-Pro-TyrΨ[CH(CN)NH]Ile-Leu-OH	**14a**	S	S	R	180	5.5
	14b	S	S	S	10000	0.1
H-Arg-Arg-Pro-Tyr-Phe-Leu-OH (**17**)^b		S	S		8	105
H-Arg-Arg-Pro-Tyr-Ile-Phe-OH (**18**)^b		S	S		5	200
H-Arg-Arg-Pro-TyrΨ[CH$_2$NH]Ile-Leu-OH (**19**)^c		S	S		6310	0.1
H-Arg-Arg-Pro-Tyr-IleΨ[CH$_2$NH]Phe-OH (**20**)^b		S	S		8000	0.1
H-Arg-Arg-Pro-Tyr-Ile-Leu-OH (NT$_{8-13}$)		S	S		10	100

^{a} Binding to rat cortex membranes using [^{3}H]NT as tracer. Values are the mean of three separate experiments, each performed in duplicate (standard errors ± 10−15%). ^{b} For comparative purposes these NT$_{8-13}$ analogues were synthesized by standard SPS procedures. For the preparation of the methyleneamino derivative **20**, see ref 9. ^{c} From ref 9.

peptide bond surrogate was carried out, *via* a modified Strecker synthesis, from Fmoc-Tyr(^{t}Bu)-H, H-Ile-Leu-(*p*-alkoxybenzyl)-resin, and (trimethylsilyl)cyanide in the presence of ZnCl$_2$. The pair of epimers **14a,b**, obtained in 43% overall yield, was separated by semipreparative HPLC. In order to establish the absolute configuration at the asymmetric center of the peptide bond surrogate in **14a,b**, the pseudotripeptide analogues **15a,b** were synthesized in solution, deprotected, and cyclized to their corresponding 2-oxoimidazolidines **16a,b** (Scheme 3). The pentacyclic derivative **16a** showed a $J_{4,5}$ value of 4 Hz, consistent with a *trans* disposition of H-4 and H-5 protons,[15,16] while in the imidazolidine **16b** this coupling constant was 8 Hz, indicating a *cis* relationship between substituents at the 4 and 5 positions. Therefore, compounds **15a** and **16a** have *R* configuration at the asymmetric center bearing the CN group, whereas derivatives **15b** and **16b** have the *S* configuration. Moreover, Fmoc deprotection of pseudotripeptides **15a,b** followed by coupling with Fmoc-Arg-(Pcm)-Arg(Pcm)-Pro-OH afforded, after total deblocking, the NT$_{8-13}$ pseudopeptide analogues **14a,b**, respectively, identical to those obtained by solid phase synthesis.

Attempts to obtain H-Arg-Arg-Pro-Tyr-IleΨ[CH(CN)-NH]Leu-OH with the peptide bond surrogate at the C-terminus were unsuccessful, due to the scission of the

[CH(CN)NH] bond, after cleavage from the resin. This scission was almost complete in H$_2$O at pH 4.5 after 24 h.[24]

Binding Assays. The NT$_{8-13}$ pseudohexapeptide analogues **12−14** were evaluated for their potency in displacing [^{3}H]NT from rat cortex membranes.[25] Results were compared to those obtained for NT$_{8-13}$ and related peptides (Table 2).

The relative affinity of the ketomethylene analogue **12a**, equipotent to NT$_{8-13}$, shows that the $\Psi[COCH_2]$ surrogate is a good substitute for the 11−12 peptide bond. This seems to indicate that the CO group is clearly implicated in the binding process, probably through the interaction with a hydrogen-bonding donor group of the NT receptor. The difference in binding affinity between the reduced peptide bond analogue **19** and the $\Psi[(R)CH(CN)NH]$ pseudohexapeptide **14a** appears to support this assumption. Thus, while compound **19** does not show appreciable affinity for the receptor, compound **14a**, in which the CN group keeps some hydrogen bond acceptor capacity, exhibits a moderate affinity. The loss of potency shown by the (*S*)-CH(CN) isomer **14b** could be explained in terms of an inappropriate spatial disposition of the CN group that prevents the formation of the hydrogen bond required for the correct interaction with the receptor. The

importance of the CO group of the 12–13 amide linkage for efficient recognition of the NT receptor was demonstrated by the approximately 66-fold differences in the binding properties between ketomethylene derivative **13a** and the reduced peptide bond analogue **20**. Thus, replacement of this peptide bond by the Ψ[COCH₂] surrogate only led to a slight decrease in the affinity, while substitution by a reduced peptide bond strongly modified the receptor binding capacity. The increased flexibility introduced at the C-terminal part of the molecule by the ketomethylene moiety could explain the decrease in affinity of pseudohexapeptide **13a** when compared to NT₈₋₁₃ and its [Phe¹³] analogue **18**, leading to a less favorable interaction of the benzyl side chain of **13a** with the hydrophobic receptor subsite corresponding to the Leu¹³ residue. Anyway, a certain participation of the NH group of the C-terminal peptide bond can not be discarded.

Compounds **12b** and **13c**, incorporating D residues at position 12, presented lower affinities than the corresponding L analogues **12a** and **13a**, in agreement with published results concerning the activity of [D-Ile¹²]-NT.[26,27] Similarly, the decrease in affinity of the [Ile¹²Ψ-[COCH₂]-D-Phe¹³]NT₈₋₁₃ analogue **13b** with respect to **13a** is also in concordance with the effect of replacing Leu¹³ with its D isomer.[26]

In summary, the biological results here presented seem to indicate that the carbonyl groups of the peptide bonds between residues 11–12 and 12–13 play an important role for the effective recognition of the central NT receptor. However, further studies are required to completely clarify the importance of the C-terminal amide bond in this binding process.

Experimental Procedures

¹H NMR spectra were recorded with a Varian Gemini-200, a Varian XL-300, a Bruker AMX2-400, or a Varian Unity 500 spectrometer, operating at 200, 300, 400, or 500 MHz, respectively. ¹³C NMR spectra were recorded with a Gemini-200 spectrometer (50 MHz). Plasma desorption MS (PD-MS) were recorded with a Bio-Ion 20 (Applied Biosystems) instrument. Elemental analyses were obtained on a CHN-O-RAPID apparatus. Amino acid analyses were performed using hydrolysis by 6 N HCl at 110 °C for 22 h and precolumn treatment with phenyl isothiocyanate followed by HPLC analysis. Analytical TLC was performed on aluminum plates precoated with a 0.2 mm layer of silica gel 60 F₂₅₄ (Merck). Silica gel 60 (230–400 mesh; Merck) was used for column chromatography. Compounds were detected with UV light and ninhydrin.

Analytical HPLC was performed on a Vydac 218TP54 C₁₈ (4.6 × 250 mm, 5 μm) or a μ-Bondapak C₁₈ (3.9 × 300 mm, 10 μm) column, respectively. The following solvent systems were used (A) CH₃CN/0.1 M (NH₄)₂SO₄ (pH = 2.5); linear gradient from 5% to 60% CH₃CN over 50 min; (B) CH₃CN/0.05% TFA, isocratic conditions. In all cases the flow rate was 1 mL/min and UV detection was 214 nm. Semipreparative HPLC was performed on a C₁₈ (25 × 250 mm, 7 μm) column, which was eluted with the solvent system A (gradient: slope of 0.2% CH₃-CN/min), at 10 mL/min. The purified peptides were desalted by absorption on C₁₈ Sep-Pak cartridges which were equilibrated with 0.1% TFA and then eluted with

70% CH₃CN/0.1% TFA. The peptides were isolated from this eluate by lyophilization after proper dilution with H₂O.

General Procedure for the Synthesis of Chloromethyl Ketones Derived from Boc-Ile-OH and Boc-Tyr(2,6-di-Cl-Bzl)-OH. *N*-Methylmorpholine (20 mmol) and isobutyl chloroformate (24 mmol) were added to a cooled solution (−20 °C) of the Boc-protected amino acid (20 mmol) in dry THF (50 mL). The mixture was stirred at that temperature for 30 min and then filtered. An ethereal solution of diazomethane, prepared from *N*-nitroso-*N*-methylurea (50 mmol), was added to the filtrate, and the reaction mixture was stirred for 15 min at 0 °C. Then, 2 N methanolic HCl was added at room temperature until N₂ evolution ceased. The solution was neutralized with TEA, and the solvents were evaporated. The resulting residue was dissolved in EtOAc (200 mL) and washed with H₂O, and the organic layer was dried over Na₂SO₄. After evaporation, the product was purified on a silica gel column using the solvent system specified in each case.

Boc-Tyr(2,6-di-Cl-Bzl)-CH₂Cl (1): yield 80%; EtOAc–hexane, 1:6. Anal. (C₂₂H₂₄Cl₃NO₄) C, H, N.

Boc-Ile-CH₂Cl (2): yield 82%; EtOAc–hexane, 1:9. Anal (C₁₂H₂₂ClNO₃) C, H, N.

General Procedure for the Synthesis of Benzyl γ-Keto Diesters 3 and 4. A mixture of chloromethyl ketone **1** or **2** (3.6 mmol) and sodium iodide (3.6 mmol) in dry THF (15 mL) was stirred at room temperature for 15 min and then added to a solution of the monosodium salt of dibenzyl malonate (4 mmol) in dry THF (10 mL). Stirring was continued for 1 h, the solvent was removed, and the residue was extracted with EtOAc (100 mL) and washed with H₂O (50 mL). The organic extract was dried (Na₂SO₄) and evaporated leaving a residue which was purified on a silica gel column, using the solvent system specified in each case.

Benzyl 5(S)-[(*tert*-butyloxycarbonyl)amino]-2-(benzyloxycarbonyl)-6-[4-[[(2,6-dichlorophenyl)-methyl]oxy]phenyl]-4-oxohexanoate (3): yield 86%; EtOAc–hexane, 1:5. Anal. (C₃₉H₃₉Cl₂NO₈) C, H, N.

Benzyl 5(S)-[(*tert*-butyloxycarbonyl)amino]-2-(benzyloxycarbonyl)-6(R)-methyl-4-oxooctanoate (4): yield 75%; EtOAc–hexane, 1:7. Anal. (C₂₉H₃₇NO₇) C, H, N.

General Procedure for the Synthesis of Benzyl 2-Substituted γ-Keto Diesters 5 and 6. A stirred solution of γ-keto diester **3** or **4** (2.8 mmol) and sodium hydride (3 mmol) in dry THF (25 mL) was treated with benzyl bromide (5.6 mmol). After 5 h of stirring at room temperature, the solvent was evaporated and the residue was extracted with EtOAc (100 mL) and washed with H₂O (50 mL). The organic layer was dried (Na₂-SO₄) and evaporated leaving a residue which was purified on a silica gel column using EtOAc–hexane (1:6) as eluent.

Benzyl 5(S)-[(*tert*-butyloxycarbonyl)amino]-2-benzyl-2-(benzyloxycarbonyl)-6-[4-[[(2,6-dichlorophenyl)methyl]oxy]phenyl]-4-oxohexanoate (5): yield 91%. Anal. (C₄₆H₄₅Cl₂NO₈) C, H, N.

Benzyl 5(S)-[(*tert*-butyloxycarbonyl)amino]-2-benzyl-2-(benzyloxycarbonyl)-6(R)-methyl-4-oxooctanoate (6): yield 73%. Anal. (C₃₆H₄₃NO₇) C, H, N.

5(S)-[(*tert*-Butyloxycarbonyl)amino]-2-benzyl-2-carboxy-6-[4-[[(2,6-dichlorophenyl)methyl]oxy]-

phenyl]-4-oxohexanoic Acid (7). A solution of the benzyl γ-keto diester **5** (1.74 g, 2.1 mmol) in EtOAc (100 mL) was hydrogenated at 30 psi and room temperature in the presence of 10% Pd−C (174 mg) for 30 min. The catalyst was removed by filtration, and the filtrate was evaporated to dryness to give 1.3 g (96%) of a white foam.

5(S)-[(tert-Butyloxycarbonyl)amino]-2-benzyl-2-carboxy-6(R)-methyl-4-oxooctanoic Acid (8). **8** was obtained in 90% yield from compound **6** (1.5 g, 2.5 mmol) following the same procedure as for the preparation of derivative **7**.

General Procedure for the Decarboxylation of the Malonic Acid Derivatives 7 and 8. Diacid **7** or **8** (1.5 mmol) was dissolved in xylene (15 mL) and heated under reflux for 3 h. Removal of the solvent left a syrup that was purified on a silica gel column using EtOAc−hexane (1:2) as eluent.

Boc-Tyr(2,6-di-Cl-Bzl)Ψ(COCH₂)(R,S)-Phe-OH (9): yield 89%; HPLC t_R = 20.72 min (μ-Bondapak, eluent system B (60/40)). Anal. (C₃₁H₃₃Cl₂NO₆) C, H, N.

Boc-IleΨ(COCH₂)(R,S)-Phe-OH (10): yield 66%; HPLC t_R = 29.01 min (**10a**) and 30.28 min (**10b**) (μ-Bondapak, eluent system B (45/55)). Anal. (C₂₁H₃₁NO₅) C, H, N.

Synthesis of 2,5-Diketopiperidine Derivatives 11a,b. A solution of compounds **10a** or **10b** (150 mg, 350 μmol) in TFA/CH₂Cl₂ (1:2, 10 mL) was stirred at 0 °C for 1 h. Then, the solvents were evaporated to dryness, and to the foam obtained, dissolved in CH₂Cl₂ (3 mL), were added at −30 °C BOP (192 mg, 420 μmol) and TEA (0.11 mL, 770 μmol) in CH₂Cl₂ (3 mL). After stirring at that temperature for 30 min, the solution was diluted with CH₂Cl₂ (134 mL) and the reaction mixture stirred at room temperature for 12 h. After evaporation, the resulting residue was extracted with EtOAc (50 mL) and washed with H₂O (30 mL), and the organic layer was dried (Na₂SO₄) and evaporated, leaving a residue which was purified on a silica gel column using EtOAc−hexane (1:2) as eluent.

3(R)-Benzyl-6(S)-(1'(R)-methylpropyl)-2,5-diketopiperidine (11a): yield 67%; HPLC t_R = 10.80 min (μ-Bondapak, eluent system B (45/55)); ¹H NMR (300 MHz, CDCl₃) δ 0.85 (t, 3H, 3'-H), 0.96 (d, 3H, 1'-CH₃), 1.28 (m, 2H, 2'-H), 1.99 (m, 1H, 1'-H), 2.27 (dd, 1H, 4-H), 2.50 (dd, 1H, 4-H), 2.66 (dd, 1H, 3-CH₂), 2.74 (m, 1H, 3-H), 3.40 (m, 1H, 3-CH₂), 3.76 (d, 1H, 6-H), 6.06 (s, 1H, 1-H), 7.13−7.28 (m, 5H, C₆H₅). Anal. (C₁₆H₂₁NO₂) C, H, N.

3(S)-Benzyl-6(S)-(1'(R)-methylpropyl)-2,5-diketopiperidine (11b): yield 46%; HPLC t_R = 12.86 min (μ-Bondapak, eluent system B (45/55)); ¹H NMR (300 MHz, CDCl₃) δ 0.84 (t, 3H, 3'-H), 0.92 (d, 3H, 1'-CH₃), 1.26 (m, 2H, 2'-H), 1.98 (m, 1H, 1'-H), 2.42 (dd, 1H, 4-H), 2.53 (dd, 1H, 4-H), 2.68 (dd, 1H, 3-CH₂), 2.86 (m, 1H, 3-H), 3.22 (dd, 1H, 3-CH₂), 3.53 (dd, 1H, 6-H), 6.17 (d, 1H, 1-H), 7.12−7.28 (m, 5H, C₆H₅). Anal. (C₁₆H₂₁NO₂) C, H, N.

Solid Phase Synthesis of Ψ[COCH₂]NT₈₋₁₃ Analogues. Preparation of Boc-Tyr(2,6-di-Cl-Bzl)-Ψ[COCH₂](R,S)Phe-Leu-PAM Resin. Compound **9** (1.6 equiv) was coupled to H-Leu-PAM resin (1 equiv) using BOP (1.5 equiv) and DIEA (3 equiv), in CH₂Cl₂/DMF (9:1) for 20 h at room temperature. The quantitative ninhydrin test indicated a coupling yield > 99%.

Preparation of Boc-IleΨ[COCH₂](R,S)Phe-resin Using the Cs Salt and Chloromethyl Poly(styrene) Resin. According to the Gisin method,[28] compound **10** (1 equiv) was dissolved in EtOH/H₂O (4:1) and neutralized to pH 7.0 with Cs₂CO₃ and the resulting solution was evaporated to dryness and stripped with toluene. Chloromethyl poly(styrene)−1% divinylbenzene resin (1.08 mequiv/g, 1 equiv) was stirred with the resulting Cs salt in DMF at 50 °C for 18 h. The resulting substitution was 0.59 mequiv/g (nitrogen content).

Preparation of Boc-IleΨ[COCH₂](R,S)Phe-resin Using DCC/DMAP and Hydroxymethyl Resin. Compound **10** (1 equiv) was added to a suspension of 0.7 equiv of hydroxymethyl resin (1.1 mequiv/g) in CH₂Cl₂. Then, DCC (1 equiv) and DIEA (0.1 equiv) were added, and the mixture was stirred for 18 h at room temperature. No capping of residual OH groups was performed. The resulting substitution was 0.24 mequiv/g (nitrogen content).

Peptide Synthesis. The synthesis of the Ψ[COCH₂]-modified peptides was finished by extending the above Ψ[COCH₂] di- and tripeptide resins using Boc SPS methodology on a 430A (Applied Biosystems) peptide synthesizer. Treatment with TFA for 5 min was used for the Nα deblocking. Neutralization of the resin was achieved with 20% DIEA in DMF for 1 min. Couplings were performed in DMF for 20 min using a 7.5-fold excess of the preformed symmetrical anhydrides of the protected amino acid derivatives Boc-Tyr(2Br-Z)-OH and Boc-Pro-OH. In the case of Boc-Arg(Tos)-OH, a 15-fold excess of the preformed HOBt ester was used. For the Ψ[COCH₂]-modified dipeptide, attached to a hydroxymethyl poly(styrene) resin, preformed HOBt esters were used for all couplings to avoid reaction with the residual hydroxy groups on the resin. Final hexapeptide derivatives were cleaved from the resin by acidolysis using HF/m-cresol (9:1) for 75 min at 0 °C. The crude peptides were precipitated with ether and collected by filtration. After extraction into 1% AcOH, the peptides were lyophilized and purified using semipreparative RP-HPLC. The purity of the final pseudopeptides was assessed by analytical HPLC, PD-MS, amino acid analysis, and ¹H NMR (Tables 3 and 4).

Solid Phase Synthesis of [Tyr¹¹Ψ[CH(CN)]-Ile¹²]NT₈₋₁₃ Analogues 14a,b. H-Ile-Leu-(p-alkoxybenzyl)-resin was synthesized from Fmoc-Leu-p-alkoxybenzyl poly(styrene)−1% divinylbenzene resin using Fmoc SPS methodology on a 430A (Applied Biosystems) peptide synthesizer. Treatment with 20% piperidine in DMF for 17 min was used for the Nα deblocking. Coupling was performed in DMF for 60 min using a 7-fold excess of the preformed HOBt ester of Fmoc-Ile-OH. Dry ZnCl₂ (27 mg, 20 μmol) and Fmoc-Tyr(ᵗBu)-H (177 mg, 400 μmol), prepared from Fmoc-Tyr(ᵗBu)-N(CH₃)OCH₃,[29] were added to a suspension of H-Ile-Leu-(p-alkoxybenzyl) poly(styrene) resin (200 μmol) in DMF (8 mL), cooled to −20 °C. The resulting suspension was stirred at that temperature for 1 h. Then, TMSCN (0.11 mL, 800 μmol) was added, and stirring was continued at 0 °C overnight. This process was repeated to give a coupling yield, according to the quantitative ninhydrin test, of 90.7%. Solid phase synthesis was then continued with Fmoc-Pro-OH and Fmoc-Arg(Mtr)-OH as above. Cleavage from the resin was performed with TFA/phenol/4-(methylthio)phenol/

Table 3. Analytical Data of Ψ[COCH₂] and Ψ[CH(CN)NH] Pseudopeptide NT$_{8-13}$ Analogues

compd	yield (%)	plasma desorp MS (M⁺ + 1)	anal. HPLC^a t_R (min)	amino acid analyses				
				Arg	Pro	Tyr	Ile	Leu
12a	14	851.4	22.2	2.21	0.94	–	–	0.87
12b	8	851.3	26.4	2.13	0.97	–	–	0.89
13a	14	851.1	24.3	1.92	1.03	0.56^b	–	–
13b	12	851.0	25.3	1.98	0.97	0.58^b	–	–
13c	15	850.9	25.9	1.98	0.92	0.58^b	–	–
13d	19	851.0	24.8	2.06	1.05	0.57^b	–	–
14a	15	829.1	22.7	1.86	1.09	–	–	0.96
14b	28	828.7	24.7	1.94	1.10	–	–	1.06

^{a} CH₃CN/0.1 M (NH₄)₂SO₄ (pH 2.5), linear gradient from 5% to 60% CH₃CN over 50 min, flow rate 1 mL/min, 214 nm UV detection. ^{b} Significant destruction during hydrolysis with 6 N HCl at 110 °C was observed.

Table 4. Significant ¹H NMR Data of Pseudopeptides 12,^{a} 13,^{a} 14,^{b} 16,^{c} and 18^c

residue	proton	12a	12b	13a	13b	13c	13d	14a	14b	15a	15b	16a	16b
Arg⁸	α-CH	4.08	4.09	4.08	4.08	4.10	4.07	3.97	3.95	–	–	–	–
Arg⁹	α-CH	4.60	4.62	4.62	4.65	4.63	4.62	4.38	4.37	–	–	–	–
Pro¹⁰	α-CH	4.35	4.34	4.37	4.38	4.42	4.40	4.32	4.32	–	–	–	–
Tyr¹¹	α-CH	4.47	4.55	4.54	4.57	4.63	4.62	4.08^d	4.12^d	3.74^d	4.50^d	3.95^d	4.01^d
Ile(Phe)¹²	α-CH	3.04	3.04	4.24	4.14	4.23	4.32	2.92	3.01	3.23	3.29	3.95	3.72
Ile¹²	γ-CH₂	–	–	1.18	1.27	0.82	0.80	1.62	1.48	1.52	1.47	1.50	1.47
									1.12	1.16	1.15	1.10	1.15
Ile¹²	γ-CH₃	–	–	0.77	0.76	0.52	0.51	0.84	0.84	0.95	0.94	0.92	0.88
Leu(Phe)¹³	α-CH	4.23	4.08	2.98	2.97	3.07	3.05	4.18	4.20	4.46	4.50	4.44	4.38
Leu¹³	γ-CH	1.54	0.92	–	–	–	–	1.70	1.59	1.64	1.62	1.65	1.62
others	COCH₂	2.83	2.72	2.70	2.69	2.88	2.70	–	–	–	–	–	–
		2.72	2.66	2.50	2.25								
	CH(CN)	–	–	–	–	–	–	3.60^d	3.55^d	4.32^d	3.81^d	4.84^e	4.52^f

^{a} Registered in D₂O. ^{b} Registered in DMSO-d_6. ^{c} Registered in CDCl₃. ^{d} In the (cyanomethylene)amino derivatives Tyr α-CH proton moves to the β-position while the α-position corresponds to the CH(CN) proton. ^{e} $J_{4,5}$ of the imidazolidine ring showed a value of 4 Hz. ^{f} $J_{4,5}$ = 8 Hz.

ethanedithiol/2-methylindole/H₂O (26:1:1:1:1:1) for 18 h at room temperature. Isolation and purification of final pseudohexapeptides were carried out as specified for the ketomethylene derivatives. Yields, analytical HPLC, PD-MS, and amino acid analysis of compounds 14a,b are recorded in Table 3. ¹H NMR significant data of these analogues are listed in Table 4.

Synthesis of Pseudohexapeptides 14a,b in Solution. Fmoc-Tyr(ᵗBu)Ψ[CH(CN)NH]Ile-Leu-OᵗBu. Fmoc-Tyr(ᵗBu)-H (0.44 g, 1 mmol) was stirred with H-Ile-Leu-OᵗBu (0.15 g, 0.5 mmol) and anhydrous ZnCl₂ (68.1 mg, 0.5 mmol) in DMF (14 mL) at −20 °C for 1 h. Then, TMSCN (0.267 mL, 2 mmol) was added, and stirring was continued at 0 °C for 24 h. After dilution with H₂O (50 mL) and extraction with EtOAc (50 mL), the organic layer was washed with 5% KHSO₄ (2 × 50 mL), 5% NaHCO₃ (2 × 50 mL), and H₂O (2 × 50 mL), dried over Na₂SO₄, and concentrated to yield the crude product as an epimeric mixture. These epimers were separated and purified by semipreparative RP-HPLC using a 55−68% gradient of 0.1% TFA in acetonitrile over 50 min.

[(R)CH(CN)NH] isomer 15a: yield 89.5 mg, 27.4%; t_R = 41.02 min (Vydac, eluent system A). Anal. (C₄₅H₆₂N₄O₆) C, H, N.

[(S)CH(CN)NH] isomer 15b: yield 134.2 mg, 41%; t_R = 48.62 min (Vydac, eluent system A). Anal. (C₄₅H₆₂N₄O₆) C, H, N. The ¹H NMR data of these compounds are recorded in Table 4.

H-Arg-Arg-Pro-TyrΨ[CH(CN)NH)]Ile-Leu-OH (14). The R and S isomers of Fmoc-Tyr(ᵗBu)Ψ[CH(CN)NH]-Ile-Leu-OᵗBu 15a,b (13 mg, 17 μmol) were separately deprotected by treatment with 20% piperidine/DMF (1 mL) for 20 min. Then the reaction was stopped by addition of AcOH (0.3 mL), and the mixture was diluted with H₂O (10 mL). The corresponding deprotected

pseudotripeptide was isolated by semipreparative HPLC of the crude reaction mixture (conditions as above) followed by repeated lyophilization in H₂O (2 × 5 mL) containing 34 μmol of HCl. Subsequently, the N-deprotected isomeric pseudotripeptides (6.0 mg) were coupled with Fmoc-Arg(Pmc)-Arg(Pmc)-Pro-OH (11.8 mg), previously synthesized using the Fmoc SPS methodology. The couplings were carried out in DMF (4 mL) using HBTU (3.8 mg, 10 μmol) in the presence of DIEA (6.8 μL, 40 μmol) for 3 h. The resulting pseudohexapeptides were isolated by precipitation in 10% MeOH/H₂O (50 mL) at pH 4 and deprotected by treatment with TFA/phenol/4-(methylthio)phenol/ethanedithiol/2-methylindole/H₂O (100:4:4:4:4:4, 0.6 mL) for 18 h at room temperature. The crude Fmoc-protected peptides were isolated by precipitation and washing with Et₂O (50 mL) followed by dissolution in H₂O and lyophilization. Finally, the Fmoc protection was removed by treatment with 20% piperidine/DMF (2.5 mL) for 15 min at room temperature. The reaction was stopped by addition of AcOH (0.5 mL), and the mixture was diluted with H₂O (25 mL). Final compounds were isolated and purified by semipreparative HPLC as previously indicated. R isomer 14a: yield 2.14 mg, 30%. S isomer 14b: yield 2.91 mg, 40%.

Synthesis of 2-Oxoimidazolidines 16a,b Derived from H-Tyr(ᵗBu)Ψ[CH(CN)NH]Ile-Leu-OᵗBu. The corresponding epimer (R or S) of Fmoc-Tyr(ᵗBu)Ψ[CH-(CN)NH]Ile-Leu-OᵗBu 15a or 15b (50 mg, 65 μmol) was stirred in a (1:1) mixture of morpholine and CH₂Cl₂ (2 mL) at room temperature for 6 h. Then, the reaction mixture was evaporated, and the crude Fmoc-deprotected pseudotripeptide was purified by flash chromatography using a 10−20% gradient of EtOAc in hexane. The resulting deprotected compound (27 mg, 77%, 51 μmol) was dissolved in dry CH₂Cl₂ (10 mL), subse-

quently, bis(trichloromethyl) carbonate (9 mg, 30 μmol) and TEA (25 μL, 180 μmol) were added, and the reaction mixture was stirred at 0 °C for 24 h. Then, after dilution with CH_2Cl_2 (10 mL), this reaction mixture was washed successively with H_2O (10 mL) and brine (10 mL), dried over Na_2SO_4, and evaporated. The crude 2-oxoimidazolidine was purified by preparative TLC using EtOAc–hexane (1:3) as eluent.

N-[2(*S*)-[4(*S*)-[[(4-*tert*-butyloxy)phenyl]methyl]-5(*R*)-cyano-2-oxoimidazolidin-1-yl]-3(*R*)-methyl-pentanoyl]-L-leucine *tert*-butyl ester (16a): yield 14.75 mg, 52%. Anal. ($C_{31}H_{48}N_4O_5$) C, H, N.

N-[2(*S*)-[4(*S*)-[[(4-*tert*-butyloxy)phenyl]methyl]-5(*S*)-cyano-2-oxoimidazolidin-1-yl]-3(*R*)-methylpen-tanoyl]-L-leucine *tert*-butyl ester (16b): yield 13.90 mg, 49%. Anal. ($C_{31}H_{48}N_4O_5$) C, H, N. The ^{1}H NMR data of these 2-oxoimidazolidine derivatives are recorded in Table 4.

Binding Assays. Binding experiments were performed on rat cortex membranes with [^{3}H]NT as described previously.[25] Incubations were carried out at 25 °C in 10 mM TES–KOH (pH 7.5), containing 1 mM EGTA-K+, 0.02% bacitracin, 1 mM benzamidine-HCl, 10 μM 1,10-phenanthroline, and 0.002% soybean trypsin inhibitor, for 60 min (0.2 mg of protein/mL). [^{3}H]NT was incubated at 5 nM in the presence of varying concentrations of the competitor. The nonspecific binding was determined in the presence of 10^{-6} M unlabeled neurotensin. Incubation was terminated by filtration through Whatman GF/B filters presoaked in 0.3% (wt/vol) poly(ethyleneimine) solution. Each filter was immediately washed three times with 4 mL of ice-cold 50 mM Tris-HCl (pH 7.4) and dried under an infrared lamp, and the radioactivity was counted by liquid scintillation.

Supplementary Material Available: ^{1}H NMR data for compounds **1–10** and ^{13}C NMR data for compound **15** (2 pages). Ordering information is given on any current masthead page.

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