Dehydro Keto Methylene and Keto Methylene Analogues of Substance P. Synthesis and Biological Activity

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The synthesis of dehydro keto methylene and keto methylene analogues of substance P using classical peptide synthesis is described. The following analogues were prepared: [pGlu^6,Gly$^9\psi$(COCH$_2$)(*RS*)Leu^{10}]SP$_{6-11}$ (**4**) and [pGlu^6,-(*RS*)Phe$^7\psi$(COCH$_2$)(*RS*)Phe^8]SP$_{6-11}$ (**8**). The use of an improved deprotection scheme employing Meerwein's reagent (Et$_3$OBF$_4$) made possible the syntheses of the novel dehydro keto methylene analogue [pGlu^6,(*RS*)Phe$^7\psi$-(COCH$_2$)Δ(*E*)Phe^8]SP$_{6-11}$ (**26**) and the tetrapeptide analogue [pGlu^6,(*RS*)Phe$^8\psi$(COCH$_2$)Gly^9]SP$_{6-9}$(-OMe) (**23**). Compound **4** was a weak agonist in provoking contractions of the guinea pig ileum. Compound **26** was a potent inhibitor of SP degradation in rat hypothalamus preparations, with an IC$_{50}$ value of 1.8 μM.

Substance P (SP), an undecapeptide whose structure is H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH$_2$, is a putative neurotransmitter with a wide variety of biological actions in the mammalian central and peripheric nervous systems.[1] Substance P has been assigned a possible role in the transmission of pain stimuli together with an interactive relationship with the enkephalins.[2-4]

Recently, it has been established that SP is only one of a family of mammalian tachykinins: the newly discovered neurokinins A and B, which are also members of this family, are structurally related compounds with a similar range of biological actions.[5,6]

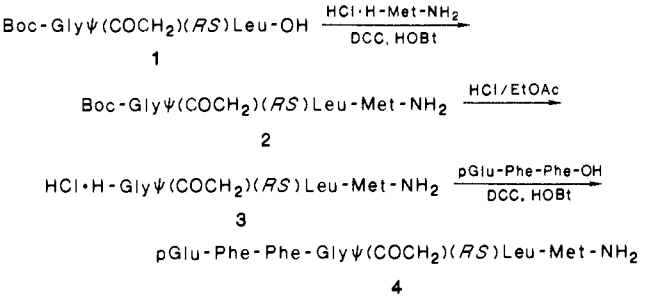
The proteolytic susceptibility of the endogenous tachykinins in the various tissue preparations results in their rapid inactivation.[7] It was shown that angiotensin converting enzyme (ACE)[8] and enkephalinase[9] both membrane-bound metalloendopeptidases which are present in nervous tissue, display high affinity for SP. In addition, a neutral membrane-bound metalloendopeptidase which specifically cleaves substance P at the Phe^8-Gly^9, Phe^7-Phe^8, and Glu^6-Phe^7 bonds, was isolated from human brains and characterized by Lee and co-workers.[10] A similar enzyme has been characterized in rat hypothalamus preparations.[11,12] The cleavage pattern of this last enzyme is parallel to the one reported by Lee et al.[10]

Metabolically stable analogues of the tachykinins have been synthesized in order to lengthen their half-life in the biological systems and thus shed light on their mode of action.

An alternative and complementary approach to the synthesis of metabolically stable analogues is the design and synthesis of specific protease inhibitors which block the enzymes responsible for inactivation. Such an approach has been applied with success to various systems such as ACE,[13-17] renin,[18,19] aspartyl protease,[20] and enkephalinase,[21] among others. The rationale of this method consists in the design of compounds which, due to their high affinity for the active site, can compete preferentially with the natural or synthetic substrate for reversible active-site occupany and, at the same time, interact strongly with functionalities present therein, thus impeding the proteolitic process.

Among the most interesting types of compounds devised for this purpose[23] are the keto methylene pseudopeptides.[24-26] Compounds of this type were found to be inhibitors of ACE[27-29] and carboxypeptidase A,[30] and they have also been used as models to study enzyme substrate interactions.[31,32]

Scheme I



Recently, too, it was disclosed that certain aminopeptidase B inhibitors of microbial origin (the so-called

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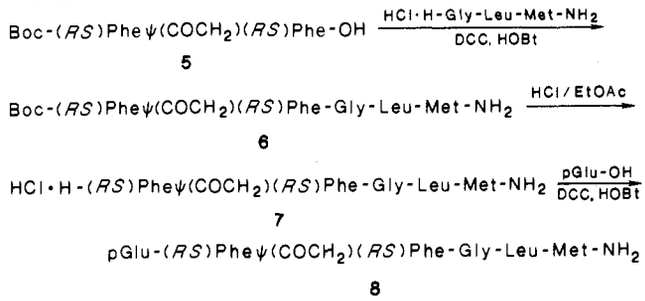
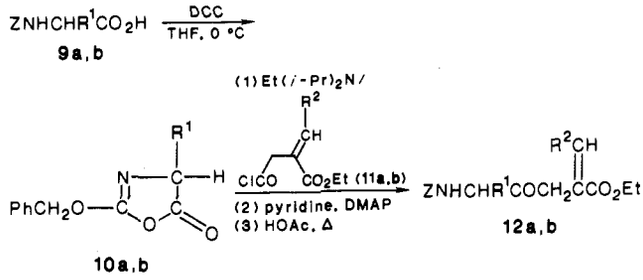
Scheme II



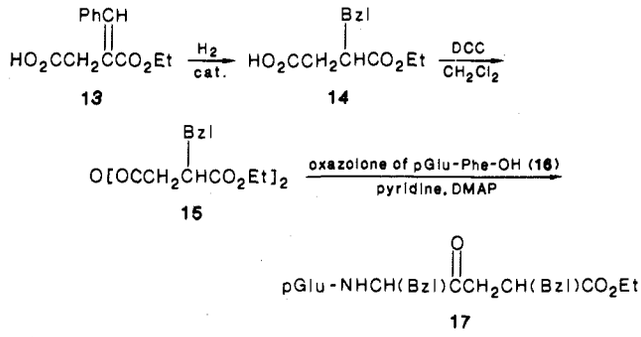
arphamenines A and B) are in fact naturally occurring keto methylene pseudodipeptide analogues of H-Arg-Phe-OH and H-Arg-Tyr-OH.[33] We have recently reported the preparation and biological properties of the SP keto methylene analogue [pGlu^6,(*RS*)Phe^{8}ψ(COCH$_2$)Gly^9]-SP$_{6-11}$.[34] This compound was a potent inhibitor of SP degradation in rat hypothalamus preparations, with an IC_{50} value of 18 μM; it was also a full agonist of SP as established in the guinea pig ileum assay, with 70% of the biological activity of the parent analogue [pGlu^6]SP$_{6-11}$, and furthermore, it was metabolically stable.[35]

Scheme III[a]

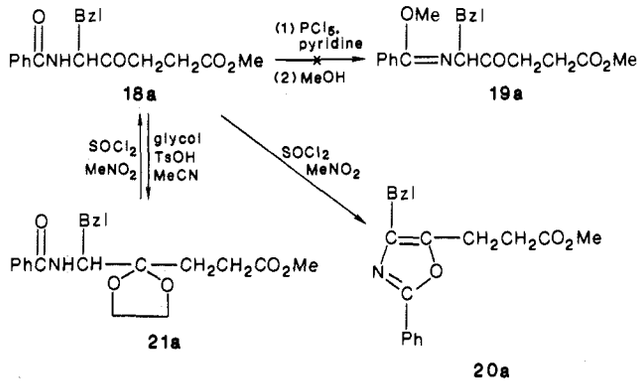


[a] Compounds 9–12: **a**, R^1 = H, R^2 = *i*-Pr; **b**, R^1 = Bzl, R^2 = Ph.

Scheme IV



Scheme V



In parallel with this, we have reported the development of a synthetic methodology for the preparation of the keto methylene pseudodipeptides.[36] On the basis of that synthetic scheme, we embarked upon the task of preparing a series of keto methylene and the novel dehydro keto methylene pseudopeptide analogues of SP with the goal of achieving inhibitors of yet greater potency. In this paper we report the synthesis of several such analogues and their biological activity. An improved synthesis is presented which increases the yield and simplifies the preparation of this type of pseudopeptide analogue.

Results and Discussion. Synthesis

The preparation of the pseudohexapeptide analogue [pGlu^6,Gly^{9}ψ(COCH$_2$)(*RS*)Leu^{10}]SP$_{6-11}$ (**4**) was achieved by following the procedure outlined in Scheme I. The *N*-Boc-protected pseudodipeptide **1** was prepared according to a previously published method.[37] This was coupled to HCl·H-Met-NH$_2$ by the use of *N*,*N*′-dicyclo-

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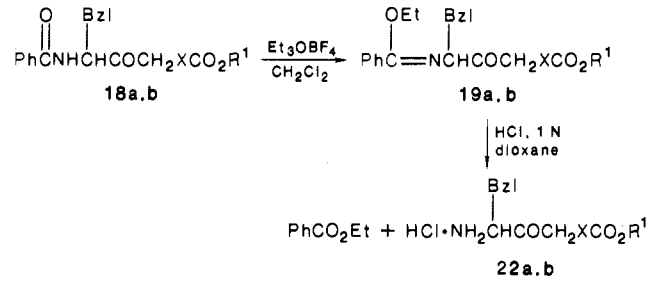
Scheme VI[a]



[a] Compounds 18, 19, 22: a, X = CH_2, R^1 = Me; b, X = C(=CHPh), R^1 = Et.

hexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBt); subsequent deprotection and coupling of **3** to pGlu-Phe-Phe-OH gave the desired analogue in acceptable yield and high purity.

Analogue **8** (Scheme II) was prepared in basically the same manner, but in this case, coupling yields were lesser. In particular, the coupling of **5** (prepared according to a previously published procedure[37]) to H-Gly-Leu-Met-NH_2 proceeded in a disappointingly slow manner, with appreciable amounts of side products. The use of 4-(dimethylamino)pyridine (DMAP) instead of HOBt produced no better results. The final product was purified by recrystallization from MeOH-H_2O.

The side reactions occurring in the course of the synthesis of analogue **8** could derive from the lower reactivity of the carboxyl group of **5** in the acylation reaction. In addition to steric factors, Wiener and Gilon[38] have suggested that the pK_a of a carboxylic acid has a strong influence on its performance in DCC-mediated acylations. It can be assumed that the pK_a of an acid such as **5** should be higher than that of the corresponding parent dipeptide, Boc-Phe-Phe-OH, and thus, lower reactivity is to be expected. Concomitantly with this, it can be assumed that the relatively unhindered free amine of the incoming Gly residue could form an imine upon reaction with the ketone carbonyl, releasing a water molecule. This, in turn, could hydrolyze the HOBt ester formed in situ, thus terminating the coupling step. This side reaction should be less prominent when residues other than Gly act as nucleophiles, due to steric reasons. It should be noted that the coupling of Boc-(*RS*)Pheψ($COCH_2$)Gly-OH to H-Leu-Met-NH_2 proceeded in a smoother way than the present one,[34] suggesting steric influences on the behavior of the carboxyl, besides the above-mentioned electronic factors. It should also be noted that couplings to the free amine of the keto methylene units proceed usually in a much smoother manner (see following discussion and ref 34).

The lower yields obtained throughout the synthesis of **8**, in addition to the complication caused by double-bond isomerization during the hydrolytic removal of the benzamido group from the precursor compound (benzoyl-(*RS*)Pheψ($COCH_2$)(Δ)Phe-OEt), prompted us to try alternative pathways for the synthesis of **8**. The alternative pathways attempted are depicted in Schemes III–VI.

Benoiton et al.[39] reported the preparation of 5-oxazolones from *N*-benzyloxycarbonyl amino acids. In our experiments, two of these compounds (**10a,b**) were generated in situ and reacted in conditions of a modified Dakin–West reaction[34,40] with the appropriately α-substituted mono-

Scheme VII

succinoyl chloride half-esters (**11a,b**).[37] This approach failed to produce the desired *N*-benzyloxycarbonyl pseudodipeptide esters **12a,b** (see Scheme III).

A recently published study reported on the synthesis of peptide ketones employing a different modification on the Dakin–West reaction.[41] On the basis of the same rationale and prior work by Steglich,[42] we attempted the preparation of the pseudotripeptide **17** (Scheme IV). To this effect, the anhydride **15** was prepared in situ from the hydrogenated product of **13**. This last compound had been prepared according to recently published methods.[37] The reaction of **15** with the oxazolone derivative of pGlu-Phe-OH **16**, generated in situ, gave a mixture of products. A medium pressure LC separation of its components showed that **17** was present in negligible amounts (as established by ^1H NMR spectra of the isolated product).

In view of the disappointing results obtained from the preceding experiments, a different approach was tried. It was felt that a mild method for the removal of the benzamido group of the precursor compound **18b** would eliminate the isomerization and concomitant yield losses. The conversion of the benzamido group of **18a,b** (Schemes V and VI) to a benzimino ether (**19a,b**) accomplishes this goal, since **19** undergoes hydrolysis under very mild conditions yielding a benzoic ester and the desired amine salt. Several reagents were studied, which could afford the imino ethers (Scheme V). Fechtig et al.[43] achieved the deprotection of β-lactam derivatives containing benzamido groups by the use of a two-step method. An imidoyl chloride is initially obtained from the amide by reaction with PCl_5; this is then attacked by MeOH as nucleophile to give the imino ether. Our attempts to achieve this imino ether by starting from **18a** failed to give the desired product **19a** (Scheme V).

Imidoyl chlorides have been prepared from amides by using different reagents[44–46] among them $POCl_3$ and $SOCl_2$. $SOCl_2$ appeared as more suitable candidate due to the gaseous byproducts obtained. Experiments in which **18a** reacted with $SOCl_2$ at room temperature yielded a solid product subsequently identified as the oxazole **20a**. This compound had previously been obtained by reaction of the same substrate with $POCl_3$ at higher temperatures.[47] In order to avoid cyclization it was reasoned that transformation of the ketone into a dioxolane (**21a** in Scheme VI) would eliminate this side reaction. Unfortunately, upon reaction with $SOCl_2$, **21a** reverted quantitatively into the starting material **18a**.

Imino ethers can be obtained directly by the reaction of amides with Et_3OBF_4 (Meerwein's salt).[48] This method has been used before to cleave amides.[49,50] In previous

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Scheme VIII

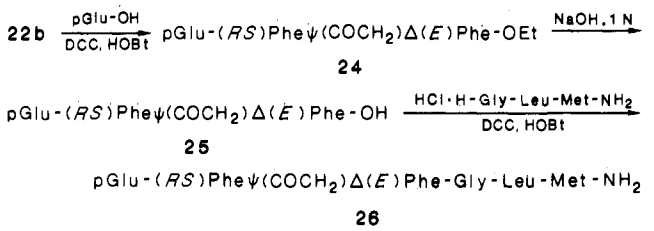


Table I. Inhibition of the Degradation of [^{125}IBH]SP$_{6-11}$ in Rat Hypothalamus Preparations by Keto Methylene and Dehydro Keto Methylene Analogues of SP[a]

no.	structure	IC_{50}, μM
4	[pGlu^6,Gly^9/ψ(COCH$_2$)Leu^{10}]SP$_{6-11}$	50
8	[pGlu^6,Phe^{7}ψ(COCH$_2$)Phe^8]SP$_{6-11}$	30
23	pGlu-Phe-Pheψ(COCH$_2$)Gly-OMe	>200
24	pGlu-Pheψ(COCH$_2$)Δ(*E*)Phe-OEt	>200
25	pGlu-Pheψ(COCH$_2$)Δ(*E*)Phe-OH	>200
18b	Bz-Pheψ(COCH$_2$)Δ(*E*)Phe-OEt	>200
26	[pGlu^6,Phe^{7}ψ(COCH$_2$)Δ(*E*)Phe^8]SP$_{6-11}$	1.8
22a	HCl·H-Pheψ(COCH$_2$)Gly-OMe	>200
22b	HCl·H-Pheψ(COCH$_2$)Δ(*E*)Phe-OEt	>200

[a] Bioassays are described in the Experimental Section.

papers, 1 equiv of this salt was necessary to effect the conversion of the amides into imino ethers.[49-51]

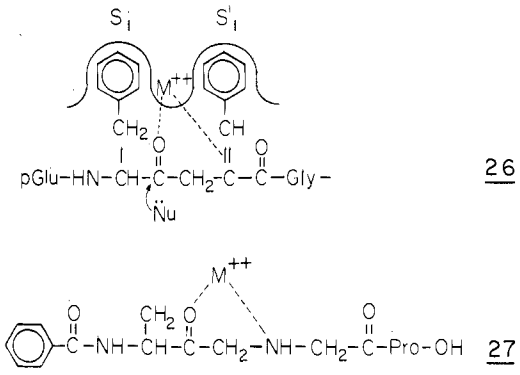
Our experiments (Scheme VI) showed that a large excess of Et_3OBF_4 (typically 6 equiv) and also higher temperatures were needed for effective conversion of the amide to the corresponding imino ether. Nevertheless, this proved to be a most convenient method for the removal of a benzamido group. Both HCl salts of the pseudodipeptide methyl esters **22a,b** were obtained in a straightforward manner, in high purity and adequate yield, by refluxing the *N*-benzoyl pseudodipeptide methyl esters **18a,b** in a 1 M CH_2Cl_2 solution of Et_3OBF_4[52] under argon, followed by dilute acid hydrolysis at room temperature. In the case of **22b**, the remaining unreacted starting material **18b** was recovered by precipitation from MeOH–H_2O, following the removal of the product which was precipitated from ether.

The characterization of **22a,b** showed that no transesterification had occurred for **22a** and, furthermore, that the double bond of **22b** had remained unchanged in the *E* configuration.

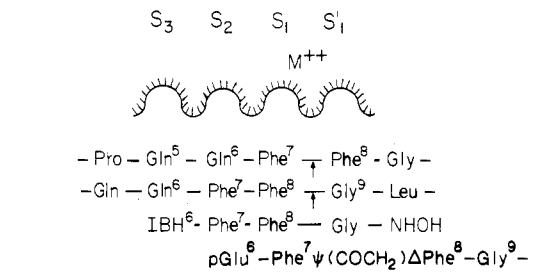
Scheme VII shows the preparation of pseudotetrapeptide analogue **23**. The synthesis of this compound by the previously used method (see Schemes I and II) would have entailed hydrolysis of **18a** and subsequent reesterification of the free carboxyl group prior to the final coupling step. The alternative pathway used here obviates this last step and improves the overall yield.

The synthesis of the novel dehydro keto methylene pseudohexapeptide **26** is described in Scheme VIII. Initially **22b** was coupled to pGlu-OH with good yield by conventional methods. The pseudotripeptide ester **24** was saponified to afford the free acid **25**. The coupling of **25** to HCl·H-Gly-Leu-Met-NH_2 was very slow and proceeded with formation of side products. It was attempted to accelerate the reaction by using DCC/DMAP. Catalytic amounts of DMAP did not improve the results. Equivalent amounts of DMAP in DMF promoted the formation of side products, conceivably due to double-bond isomerization. The analogue **26** was eventually synthesized by

Scheme IX



Scheme X



using DCC and HOBt in DMF, and the crude product was purified by semipreparative HPLC.

Biological Activity

A fast screening method which was recently developed[53] allowed us to evaluate the inhibitory activities of the analogues prepared in the course of this study. Table I summarizes the values obtained using the different analogues as inhibitors of the degradation of *N*-[3-(3-[^{125}I]-iodo-4-hydroxyphenyl)propanoyl]SP$_{6-11}$ ([^{125}IBH]SP$_{6-11}$) in rat hypothalamus slice preparations. Analogue **26** was the most potent inhibitor of the series. Other hexapeptide analogues prepared in this work were less potent, and the shorter pseudopeptides showed still lower affinity for the enzyme.

The different IC_{50} values of keto methylene analogue **8** and dehydro keto methylene analogue **26** could be explained by two different tentative models describing the mode of interaction of these analogues with the active site of the enzyme. The first model is outlined in Scheme IX. In this model, the dehydro keto methylene moiety of compound **26** would provide a bidentate ligand for the metal in the active site. A comparison between this mode of interaction and that of the keto methylene ACE inhibitor described by Gordon et al.[54] implied that the nitrogen in the secondary amine of compound **27** (Scheme IX) conceivably mimics the situation of the amide nitrogen during enzymatic cleavage by undergoing protonation by one of the residues in the active site. This in turn would render the ketone more prone to nucleophilic attack, for example, by a water molecule, thus facilitating binding to the metal atom.

As presented in our tentative model shown in Scheme IX, an alternative binding mode could be envisaged in which the ketone carbonyl and the secondary amine of **27** (which are positioned relative to one another in a similar manner as the double bond and the ketone functions of

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26) would embody the prongs of a bidentate metal ligand. This suggestion appears to be quite consistent with the findings of Gordon et al.[54] and with those of Natarajan et al.[55]

Alternatively, the better inhibitory potency of dehydro analogue **26** compared to the reduced analogue **8** could be explained by a more favorable side-chain geometry, causing a better fit in the corresponding hydrophobic pocket S'_1 (See Scheme X). Different patterns of interaction can likewise be attributed to analogue **26** and to another known potent SP-degradation inhibitor, *N*-[3-(3-iodo-4-hydroxyphenyl)propanoyl]-Phe-Phe-Gly-NHOH (IBH-Phe-Phe-Gly-NHOH).[56] In this last compound, the hydrophobic residues in positions 6, 7, and 8 would fit into pockets S_3, S_2, and S_1, respectively, while the hydroxamic acid function would chelate the metal as shown in Scheme X. Further study is necessary to determine which binding mode is more realistic.

Guinea pig ileum contraction assays performed with the analogues prepared in this work showed that only compound **4** possessed agonistic properties, as it had 0.5% of the potency of the parent compound [pGlu^6]SP$_{7-11}$ in that assay; furthermore, the former compound did not show selectivity toward any of the known tachykinin receptor subclasses.[57] All other compounds were devoid of agonistic features in these experiments.

These results are in accordance with previous findings concerning the importance of certain bonds of the SP sequence in agonist–receptor interactions. Among those, modification of the parent structure in which the Phe^7-Phe^8 amide bond was substituted by a retro–inverso surrogate caused the loss of biological activity. Furthermore, N-methylation of that bond produced an analogue with high selectivity toward the SP N receptor subtype.[57]

In summary, the results presented in this paper attest to the synthetic feasibility of a novel type of pseudopeptide analogue, namely, the keto methylene, α,β-dehydro modification, and to the potential usefulness of it in the development of selective peptidase inhibitors and generally in the field of backbone modified peptide analogues.

The novel SP-degradation inhibitor **26** could foreseeably be a useful tool in the quest for the clarification of the role and mode of action of that neuropeptide in particular and that of the tachykinins in general.

Experimental Section

Melting points were measured with a Thomas-Hoover capillary melting point apparatus. TLC was run on silica gel coated plastic sheets (silica gel 60 F254, layer thickness 0.2 mm) from E. Merck, Darmstadt.

The elution systems used for TLC were (A) CH_2Cl_2–MeOH, 9:1; (B) CH_2Cl_2–MeOH, 4:1; (C) EtOAc–hexane, 2:1; and (D) 1-butanol–pyridine–1.0% HOAc in H_2O, 5:3:11 (upper phase). The detection methods employed for TLC analysis were as follows: (I) exposure of the developed plates to UV light at 254 nm; (II) spray solution of 1% ninhydrin in MeOH; (III) (a) spray solution of 1% *t*-BuOCl in cyclohexane; (b) spray solution consisting of a 1:1 mixture of (b1) 0.05 M *n*-Bu_4NI in acetone and (b2) 1% *o*-tolidine in MeOH; (IV) spray solution of 0.1% fluorescamine in acetone.

HPLC was performed on a Spectra Physics SP8000 or a Merck-Hitachi 655A system using a variable-wavelength UV detector set at 220 nm. The column used was Hibar Lichrosorb RP8 (0.4 × 25 cm).

NMR spectra were taken with a Bruker WP200 (200 MHz) or a WH300 (300 MHz) FT spectrometer. The internal standard was TMS.

Mass spectroscopy was performed by Dr. Klaus Eckhart at the laboratory of Prof. Helmut Schwarz at the Technische Universitaet, Berlin, using a FAB ion source.

Amino acid analysis was performed with an LKB 4400 amino acid analyzer, using a Spectra Physics 4100 printer plotter computing integrator. The analyses were run by using a standard 54-min program and four component sodium buffer systems.

The samples for amino acid analysis weighed about 1 mg, and they were hydrolyzed in constant boiling HCl or a mixture of $MeSO_3H$–propionic acid–H_2O at 120 °C for 20 h after degassing and sealing. After hydrolysis they were dried at high vacuum over KOH pellets and dissolved in Na citrate buffer (0.2 mL), pH 2.2. Elemental analyses were carried out at the Microanalytical Laboratory of the Institute of Chemistry, The Hebrew University, Jerusalem.

Solvent and Reagent Purification. EtOAc was stirred over K_2CO_3 and distilled therefrom. DMF was distilled under reduced pressure with vigorous stirring from fresh Al_2O_3. *N*-Methylmorpholine (NMM) was distilled from KOH. Other solvents were distilled prior to use.

Phosphoramidon was purchased from the Peptide Research Institute (Osaka, Japan), captopril was graciously provided to us by Dr. M. A. Ondetti (Squibb Institute, Princeton, NJ), and [^{125}IBH]SP$_{8-11}$ was prepared according to previously published procedures.[56]

Triethyloxonium tetrafluoroborate (Et_3OBF_4) was purchased from Fluka AG, Switzerland, as a 1 M solution in CH_2Cl_2 and was used without further treatment.

General Coupling Procedure. In an ice-cooled flask under argon, the amine hydrochloride (1 equiv), the *tert*-butyloxycarbonyl (Boc) protected amino acid or peptide (1 equiv), and hydroxybenzotriazole (HOBt) (2 equiv) were dissolved in dimethylformamide (DMF). Dicyclohexylcarbodiimide (DCC) (1 equiv) dissolved in the same solvent was cooled and added to the reaction mixture immediately followed by NMM (1 equiv). The mixture was usually allowed to reach room temperature and was stirred overnight. Dicyclohexylurea (DCU) was then filtered off, the filtrate was cooled on an ice bath, and a saturated solution of $KHCO_3$ or $NaHCO_3$ was added (4–6 times the volume of DMF). Then brine was added (about half the amount of DMF), and the resulting precipitate was collected on a sinter glass filter and washed with water until no more Cl^- was detected with an $AgNO_3$ test. At this point the solid collected was dried overnight over P_2O_5 at high vacuum. The mother liquor was usually stored for a few weeks at 4 °C, and any further precipitates were collected, washed, dried, and characterized.

General Deprotection Procedure. Boc-protected intermediates were dissolved in ethyl acetate (EtOAc) saturated with HCl. The mixture was protected from moisture with a $CaCl_2$ trap and magnetically stirred for 0.5–1 h at room temperature. Evaporation of the solvent with a reduced-pressure rotary evaporator was followed by stirring of the obtained residue under ether and decantation of the solvent. This was repeated twice more, and then the remaining residue was left to dry at high vacuum over KOH pellets.

Boc-Glyψ(COCH$_2$)(*RS*)Leu-Met-NH$_2$ (2). Compound **1** (0.7 g, 2.43 mmol) and HCl·H-Met-NH$_2$ (0.44 g, 2.43 mmol) reacted according to the general coupling procedure in 60 mL of DMF. The product isolated was recrystallized from EtOAc–petroleum ether, yielding 0.28 g (27%) of product, mp 74–76 °C dec. The mother liquor yielded a further crop of product, mp 74–76 °C dec, weighing 0.3 g (29%): TLC R_f 0.65 (A), 0.8 (B), 0.66 (D); HPLC k' 3.34 gradient from acetonitrile (MeCN) (5%)–H_2O (95%) to MeCN (40%)–H_2O (60%), flow 1 mL/min. This and subsequent gradients were developed for 20 min. Anal. ($C_{19}H_{35}N_3O_5S$) C, H, N.

pGlu-Phe-Phe-Glyψ(COCH$_2$)(*RS*)Leu-Met-NH$_2$ (4). The pseudotripeptide **2** (0.25 g, 0.59 mmol) was deprotected by using the standard deprotection method. The product **3** weighed 0.16 g (76%). This compound (0.45 mmol) was coupled to pGlu-Phe-Phe-OH (0.19 g, 0.45 mmol) by using the general coupling

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method (DMF: 10 mL). The product of this reaction was isolated from the reaction workup mixture in three crops over a period of 1 week in cold storage. The composition of the crops was the same; in total were isolated 0.11 g (34%) of product: mp 126–130 °C; HPLC k' (a) 6.32, (b) 7.01 gradient from MeCN (25%)–H_2O (75%) to MeCN (60%)–H_2O (40%), flow 1 mL/min. Peaks a and b were of similar size. They were attributed to diastereomers. Anal. ($C_{37}H_{50}N_6O_7S\cdot 2H_2O$) C, H, N. Amino acid analysis: Met, 1; Glu, 1; Phe, 2; X, 1 (the pseudodipeptide X was detected with a retention time similar to that of Lys in the standard amino acid analysis reference mixture).

Boc-(*RS*)Pheψ($COCH_2$)(*RS*)Phe-Gly-Leu-Met-NH_2 (6). The Boc-protected pseudodipeptide **5** (0.32 g, 0.92 mmol) was coupled to HCl·H-Gly-Leu-Met-NH_2 according to the general coupling procedure in CH_2Cl_2 (8 mL) and DMF (1 mL). After DCU filtration, the CH_2Cl_2 was evaporated and the DMF solution of product was treated as described above. The collected precipitate was dried, weighing 0.18 g (27.5%) of crude product: mp 102–108 °C; TLC R_f 0.72 (A); HPLC k' 4.18 MeOH (70%)–H_2O (30%), flow 1 mL/min. Amino acid analysis: Gly, 1; Leu, 1; Met, 1; X, 1 (the free pseudodipeptide X was detected at a retention time similar to that of Lys in the standard amino acid analysis reference mixture).

pGlu-(*RS*)Pheψ($COCH_2$)(*RS*)Phe-Gly-Leu-Met-NH_2 (8). The protected pseudopentapeptide **6** (50 mg, 0.07 mmol) was deprotected in the usual manner. The amine hydrochloride which was obtained, **7** (38 mg, 0.06 mmol), was coupled to pGlu-OH (7.5 mg, 0.06 mmol) by using the standard coupling procedure, in CH_2Cl_2 (5 mL). The yield of product after recrystallization from EtOAc–petroleum ether was 3.2 mg (7.6%): mp 102–106 °C; HPLC k' 3.35 MeCN (38%)–H_2O (62%), flow 1 mL/min; FAB MS, m/e 723 $[M + H]^+$. Amino acid analysis: Met, 1; Leu, 1; Gly, 1; Glu, 1; X, 1 (the free pseudodipeptide unit X was detected at a retention time similar to that of Lys in the standard amino acid analysis reference mixture).

Methyl 6-Phenyl-5-amino-4-oxohexanoate Hydrochloride (22a). Compound **18a** (0.88 g, 2.59 mmol) and Et_3OBF_4 (15 mL, 15 mmol, 1 M commercial solution in CH_2Cl_2) were stirred at 60 °C in a dry reflux system under Ar for 3 days. The solution was cooled, water was added, and the layers were separated. The organic phase was extracted with water once again. The aqueous phase was treated with a saturated solution of $NaHCO_3$ to pH 8. Then the basic aqueous solution was extracted into EtOAc, and the organic layer was twice extracted with a 1 N HCl solution. The aqueous layer was evaporated under reduced pressure and dried under high vacuum over KOH pellets. The product was obtained as a thick oil weighing 0.39 g (55.7%): TLC R_f 0.59 (A), 0.12 (C); NMR (DMSO-d_6) δ 8.42 (s, 3 H, NH_3^+), 7.33 (m, 5 H, C_6H_5), 4.36 (m, 1 H, α-C*H*, Phe), 3.62 (s, 3 H, CH_3), 3.47 (s, H_2O), 3.19 (m, 1 H, β-CH_2, Phe, $^1/_2$), 2.73 (m, 3 H, $COCH_2$, β-CH_2, Phe, $^1/_2$), 2.45 (m, DMSO), 2.38 (m, 2 H, $CH_2CO_2CH_3$). Anal. ($C_{13}H_{18}NO_3Cl\cdot 2H_2O$) C, H, N.

Ethyl 6-Phenyl-5-amino-4-oxo-2(*E*)-benzylidenehexanoate Hydrochloride (22b). The fully blocked dehydro keto methylene pseudodipeptide **18b** (3.79 g, 8.59 mmol) and Et_3OBF_4 (50 mL, 50 mmol; see **22a**) reacted as described for **22a**, for 16 h. The organic phase was extracted with water and the solvent was removed by evaporation. The residue was stirred at 25 °C with a 0.1 N HCl solution (5 mL) and dioxane (10 mL) for 1 h, the dioxane was evaporated, and the product was taken up in water–CH_2Cl_2. After workup as above, the product was mixed with ether and the resulting precipitate was collected and desiccated at high vacuum over KOH pellets: product weight, 1.74 g (54%); mp 135–139 °C; TLC R_f 0.58 (A), 0.11 (C); HPLC k' 4.38 MeOH (70%)–H_2O (30%), flow 1 mL/min; NMR (DMSO-d_6) δ 8.49 (s, 3 H, NH_3^+), 7.80 (s, 1 H, *H*-vinyl), 7.31 (m, 10 H, 2 × C_6H_5), 4.49 (m, 1 H, α-C*H*, Phe), 4.17 (s, H_2O), 4.13 (q, CH_2-ethyl, partly covered by H_2O), 3.76 (2 d, J = 19.4 Hz, superimposed $COCH_2$), 3.28 (m, 1 H, β-CH_2, Phe, $^1/_2$), 3.17 (m, 1 H, β-CH_2, Phe, $^1/_2$), 2.45 (m, DMSO), 1.20 (t, 3 H, J = 7.07 Hz, CH_3, ethyl). Anal. ($C_{21}H_{24}NO_3Cl\cdot 0.5H_2O$) C, H, N.

pGlu-(*RS*)-Pheψ($COCH_2$)Δ(*E*)-Phe-OEt (24). Compound **22b** (0.74 g, 1.97 mmol) and pGlu-OH (0.25 g, 1.97 mmol) reacted in DMF (10 mL) according to the standard coupling method. The product which was isolated in the usual manner weighed, after drying, 0.79 g (89%): mp 110–116 °C; HPLC k' 2.95 MeOH (70%)–H_2O (30%), flow 1 mL/min; NMR ($CDCl_3$) δ 7.96 (s, 1 H, *H*-vinyl), 7.35 (m, 5 H, C_6H_5), 7.26 (s, $CHCl_3$), 7.15 (m, 5 H, C_6H_5), 6.86 (d, 0.5 H, N*H*, Phe, $^1/_2$), 6.76 (d, 0.5 H, N*H*, Phe, $^1/_2$), 6.14 (s, 0.5 H, N*H*, pGlu, $^1/_2$), 5.87 (s, 0.5 H, N*H*, pGlu, $^1/_2$), 4.98 (m, 1 H, α-C*H*, Phe), 4.18 (q, 2 H, CH_2, ethyl), 4.08 (m, 1 H, α-CH, pGlu), 3.70 (s, 2 H, $COCH_2$), 3.27 (m, 1 H, β-CH_2, Phe, $^1/_2$), 2.93 (m, 1 H, β-CH_2, Phe, $^1/_2$), 2.14 (m, 4 H, $COCH_2CH_2$, pGlu), 1.33 (t, 3 H, CH_3, ethyl). Anal. ($C_{26}H_{28}N_2O_5\cdot 2H_2O$) C, H, N.

pGlu-Phe-(*RS*)Pheψ($COCH_2$)Gly-OMe (23). Compound **22a** (0.34 g, 1.27 mmol) and pGlu-Phe-OH (0.35 g, 1.27 mmol) reacted according to the general coupling scheme in DMF (10 mL). The dry isolated product weighed 0.17 g (27%) after recrystallization from EtOAc–petroleum ether: mp 91–95 °C; HPLC k' (a) 3.15, (b) 3.66 gradient from MeCN (30%)–H_2O (70%) to MeCN (70%)–H_2O (30%), flow 1 mL/min. Anal. ($C_{27}H_{31}N_3O_6$) C, H, N.

pGlu-(*RS*)Pheψ($COCH_2$)Δ(*E*)-Phe-Gly-Leu-Met-NH_2 (26). Compound **24** (96 mg, 0.21 mmol) was dissolved in dioxane (1 mL). Water was added (0.6 mL) followed by a 1 N solution of NaOH (0.43 mL). The mixture was stirred for 3 h at room temperature. The solution was partially evaporated at reduced pressure, and the residue was washed once with EtOAc and acidified to pH 2 by the addition of $KHSO_4$. The aqueous mixture was extracted three times with EtOAc. The organic layer was dried over $MgSO_4$ and evaporated under reduced pressure, and the residue was recrystallized from EtOAc–hexane. The acid obtained (**25**) weighed 70 mg. This compound was coupled to HCl·H-Gly-Leu-Met-NH_2 (60 mg, 6.17 mmol) in DMF (1.8 mL) by using the general coupling procedure. The product was purified by semipreparative HPLC on a Merck Hibar Lichrosorb RP8 column (7-μm mean particle diameter, 1.0 × 25 cm) eluted with MeOH (60%), 0.05% TFA in H_2O (40%) at 4 mL/min: HPLC k' 7.58 MeCN (38%)–H_2O (62%), flow 1 mL/min; FAB MS, m/e 721 $[M + H]^+$. Amino acid analysis: Met, 1; Leu, 1; Gly, 1; Glu, 1; X, 1 (the pseudopeptide unit X was detected at a retention time similar to that of His in the standard amino acid analysis reference mixture).

Biological Assays. Isolated Guinea Pig Ileum Assay. This was done as previously described.[11]

Rat Diencephalon Membrane System. Diencephalons from seven male albino rats were homogenized in 25 mL of ice-cold Hepes buffer (50 mM, pH 7.4) with 10 strokes of a Teflon–glass homogenizer at 800 rpm and centrifuged at 1000*g* for 10 min. The supernatant was recentrifuged at 17000*g* for a further 10 min. The resulting pellet was suspended with a Dounce homogenizer in cold buffer and centrifuged again at 17000*g* for 10 min. The final pellet was washed with cold buffer and resuspended in 5 mL of buffer (1–3 mg/mL of protein).

Assay of Peptidase Acting on the C-Terminal Sequence of Substance P. The degradation of N^{α}-(desamino-3-[^{125}I]-iodotyrosyl)SP_{6-11} and its inhibition by the above-mentioned compounds by rat diencephalon membrane preparations were assayed as previously described.[56] High concentrations (10^{-6} M each) of phosphoramidon and captopril, potent inhibitors displaying affinities in the nanomolar range toward angiotensin converting enzyme and enkephalinase, respectively[58,13] were included in the assay in order to detect SP-degrading activities distinct from these two enzymes. The IC_{50} values for all synthetic analogues were determined as described before.[53]

Acknowledgment. This research was supported by Grant GER/260 (DN 125) from the Joint German (DMFT) Israeli (NCRD) Projects administrated by G.S.F. Munchen. We thank Emma Rosenkovich and J. Frey for technical assistance.

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