vacuo (4.65 g), and a portion of this material (1.00 g) was treated with 15 mL of anhydrous HF in the presence of 10% anisole for 60 min at 0 °C. After evaporation of the HF and anisole in vacuo, the dried product was washed, under a stream of N_2, with three 30-mL portions of EtOAc and extracted with three 60-mL portions of 30% HOAc. The combined extracts were lyophilized to give 290.3 mg of Ac-[half-Cys(SH)^4,N^i-For-Trp^9,half-Cys(SH)^{10}]-α-MSH_{4-13}-NH_2.

The N^i-For-Trp disulfhydryl peptide was deformylated in the same manner as above. The deformylated peptide solution was diluted with 500 mL of 10% HOAc and the pH adjusted to 8.5 with concentrated NH_4OH. Cyclization was achieved via oxidation with 0.01 N $K_3Fe(CN)_6$ (44 mL, 100% excess) for 1 h at 25 °C. The reaction was terminated by the addition of 30% HOAc to a final pH of 5.0. Excess ferro- and ferricyanide ions were removed by addition of Rexyn 203 (Cl^- form). After filtration, the solution was lyophilized to give 1.28 g of crude Ac-[Cys^4,Cys^{10}]-α-MSH_{4-13}-NH_2. A portion of the crude peptide (310 mg) was dissolved in 3 mL of 0.01 N NH_4OAc (pH 4.5) and chromatographed on carboxymethylcellulose under the same conditions as above. The major peak eluted during the 0.1 N NH_4OAc (pH 6.8) fraction and was lyophilized to give 11.03 mg of white powder. The ion exchange was repeated on the remaining crude peptide to give a total yield of 38.9 mg. This material was then chromatographed on Sephadex G-25 (2.0 × 36.5 cm) with 0.2 N HOAc and gave one symmetric peak (280 nm detection), which was collected and lyophilized to give 18.6 mg of white, fluffy powder. The purified peptide gave single uniform spots on TLC: R_f 0.13 (A), 0.42 (B), 0.52 (C), 0.54 (D). Amino acid analysis gave the following molar ratios: Glu, 1.00; His, 0.93; Phe, 0.98; Arg, 1.04; Trp, 1.02; half-Cys, 1.94; Lys, 1.00; Pro, 0.96; Val, 1.06. Paper electrophoresis (centimeters from origin): 12.1; α-MSH, 11.5. FAB mass spectrum: MH^+ calcd, 1345; found, 1345.

Frog and Lizard Skin Bioassays. The biological activities of α-MSH and the cyclic analogues were determined by their ability to stimulate melanosome dispersion in vitro by the frog and lizard bioassays as previously described.[15,28,29] The frogs (*Rana pipiens*) used in these studies were obtained from Lemberger Co., Germantown, WI, and the lizards (*Anolis carolinensis*) were from the Snake Farm, La Place, LA.

Acknowledgment. This work was supported, in part, by grants from the U.S. Public Health Service (AM 17420) and the National Science Foundation (PCM 77-07051). We thank Dr. Karl H. Schram and Debbie Slowikowski of the University of Arizona College of Pharmacy for obtaining the FAB mass spectra. The authors also acknowledge the technical assistance of Mary E. Fealey.

Registry No. II, 81854-62-8; III, 82219-24-7; IV, 83877-16-1; V, 83897-18-1; N^α-Boc-N^i-For-Trp, 47355-10-2; N^α-Boc-N^g-Tos-Arg, 13836-37-8; N^α-Boc-Phe, 13734-34-4; N^α-Boc-N^{im}-Tos-His, 35899-43-5; N^α-Boc-γ-Bzl-Glu, 13574-13-5; N^α-Boc-(*S*-3,4-Me_2Bzl)-Cys, 41117-66-2; Ac-[half-Cys(SH)^4,N^i-For-Trp^9,half-Cys(SH)^{10}]-αMSH_{4-10}-NH_2, 83877-18-3; N^α-Boc-Pro, 15761-39-4; N^α-Boc-N^ϵ-2,4-Cl_2-Z-Lys, 42294-64-4; Ac-[half-Cys(SH)^4,*N*-For-Trp^9,half-Cys(SH)^{10}]-α-MSH_{4-13}-NH_2, 83877-19-4.

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Synthesis of Partially Modified Retro-Inverso Substance P Analogues and Their Biological Activity†

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Partial retro-inverso modification of a single peptide bond was applied to pGlu-Phe-Phe-Gly-Leu-Met-NH_2 (I), a C-terminal hexapeptide analogue of the neuropeptide substance P. Two analogues with reversed peptide bonds, between the pGlu-Phe and Phe-Gly residues, were prepared, purified and characterized. The analogue gpGlu-(*RS*)-mPhe-Phe-Gly-Leu-Met-NH_2 (II) was devoid of either agonistic or antagonistic activity. The second pseudopeptide analogue, i.e., pGlu-Phe-gPhe-mGly-Leu-Met-NH_2 (III), was found to be a full agonist with 22% of the potency of I in the guinea pig ileum assay.

Substance P (SP) is an undecapeptide that is widely distributed in the central and peripheral nervous system. It was isolated and purified to homogeneity by Chang and Leeman,[1] and its sequence was established to be the following:[2]

H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2

Synthetic SP has been prepared by Tregear et al.[3] and proved to be identical with the endogenous material.

SP belongs to the class of tachykinin-like peptides. Some of its pharmacological activities are vasodilation and spasmogenic activity,[4] salivation,[5] release of histamine from mast cells,[6] and depolarization of spinal motoneurons.[7] SP is rapidly degraded by various enzymes present in different brain preparations[8-10] and in plasma.[11,12] The major sites

† Abbreviations according to IUPAC–IUB Commission (1972), *Biochemistry*, **11**, 1726–1732, and Specialist Periodical Reports, "Amino Acids, Peptides and Proteins", Volume 11 (The Chemical Society, London, 1980, R. C. Sheppard, Ed.), are used throughout. The following special abbreviations for the partially modified retro-inverso peptides are used: The standard three-letter notation for amino acid residues preceded by the prefix g represents the *gem*-diamino alkyl residue derived from the specified amino acid. The prefix m represents the malonic acid residue derived from the amino acid specified by the three-letter notation. Configurational designation of the retro-inverso residues follows those of the amino acids.

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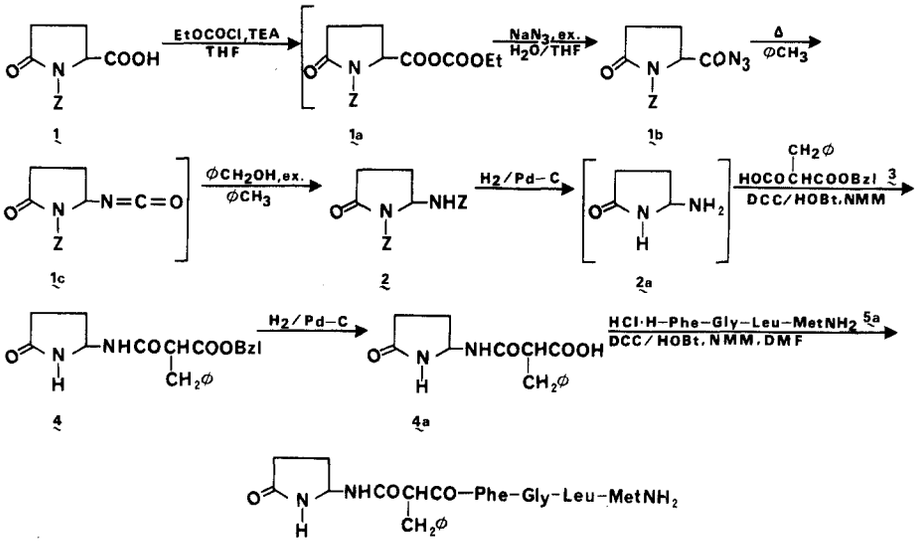
Scheme I. Synthesis of [gpGlu^6,(*RS*)-mPhe^7]SP$_{6-11}$ (II)



II

of cleavage were the peptide bonds between Gln^6-Phe^7, Phe^7-Phe^8, and Phe^8-Gly^9.[13-16] The rapid degradation of SP hampers detailed analysis of its functions and modes of action.

Two studies reported the enhancement of the metabolic stability of SP analogues in which scissible peptide bonds were N-methylated.[17,18]

A novel topochemical modification of linear peptides, the partially modified retro-inverso modification (PMRI), has been recently introduced by Goodman and Chorev.[19] This approach involves the reversal of one or more peptide bonds while maintaining the rest of the molecule intact. This topochemical modification attempts to retain at large the extended conformation of biologically active peptides. The PMRI modification was found to yield highly potent, reversal of several analogues of enkephalinamide.[20]

Figure 1 illustrates schematically the consequences of such a modification, which results in the incorporation of two non amino acid residues replacing the original amino acids. A *gem*-diamino residue results from rearrangement of the amino acid residue contributing the carbonyl function of the peptide bond undergoing reversal. A malonyl residue replaces the amino acid residue that contributes the NH group of the peptide bond undergoing

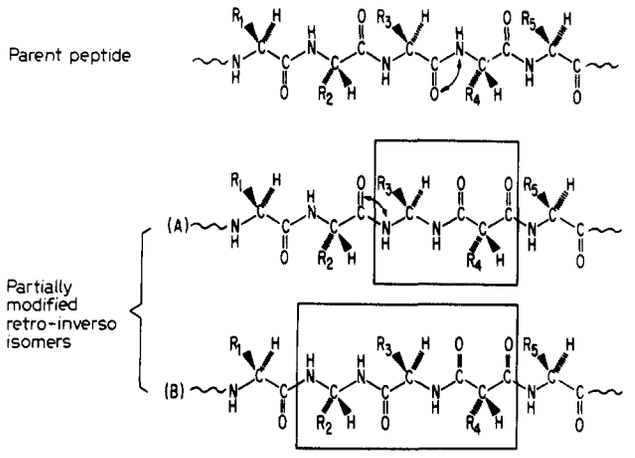


Figure 1. Schematic representation of partial retro-inverso modifications of a parent peptide. Modification A includes reversal of the peptide bond between residues R_3 and R_4, whereas modification B includes also reversal of the peptide bond between residue R_2 and R_3. Modified segments are enclosed in frames.

reversal. In the case where the PMRI analogue results from reversal of several consecutive peptide bonds, amino acids of opposite configuration and in the reversed orientation are incorporated between the *gem*-diamino and the malonyl residues.

In this paper we report the synthesis of two PMRI analogues of [pGlu^6]SP$_{6-11}$ (I). This C-terminal hexapeptide (I) derived from SP is more potent than SP in contracting the guinea pig ileum[21] and in depolarizing spinal cord motoneurons[22] and of similar potency to SP in inducing K^+ release from rat parotid slices.[18]

Each of the analogues reported here represent a reversal of a single peptide bond, which consequently results in the replacement of two adjacent amino acids by two non amino acid residues. The reversed peptide bonds were those that were found to be the most susceptible to proteolytic cleavage. Reversal of the peptide bond pGlu^6-Phe^7 resulted in analogue II and the reversal of the peptide bond

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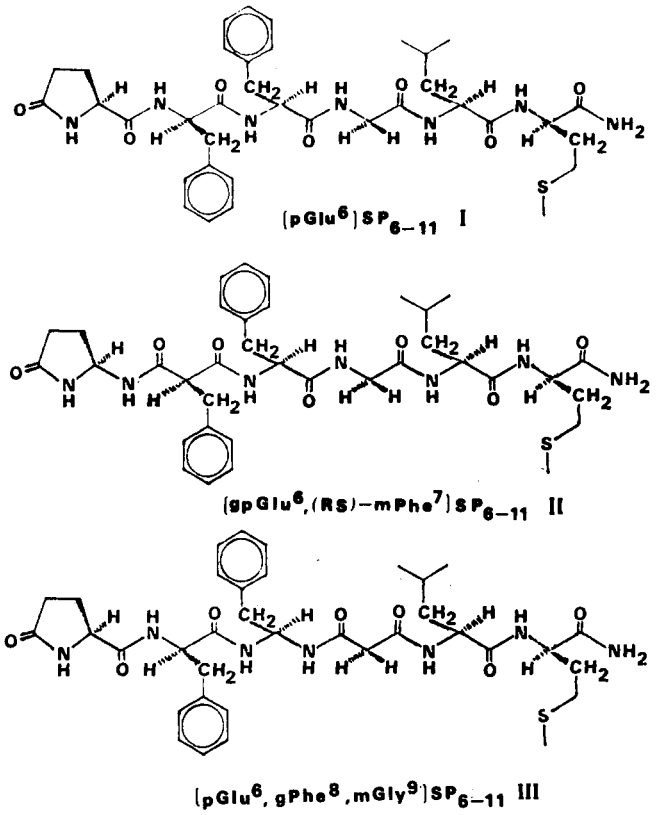


Figure 2. Structures of the parent peptide [pGlu^6]SP$_{6-11}$ (I) and its two PMRI analogues [gpGlu^6,(*RS*)-mPhe^7]SP$_{6-11}$ (II) and [pGlu^6,gPhe^8,mGly^9]SP$_{6-11}$ (III).

Phe^8-Gly^9 resulted in analogue III. The structures of the two PMRI analogues, namely, [gpGlu^6,(*RS*)-mPhe^7]SP$_{6-11}$ (analogue II) and [pGlu^6,gPhe^8,mGly^9]SP$_{6-11}$ (analogue III), are schematically represented in Figure 2.

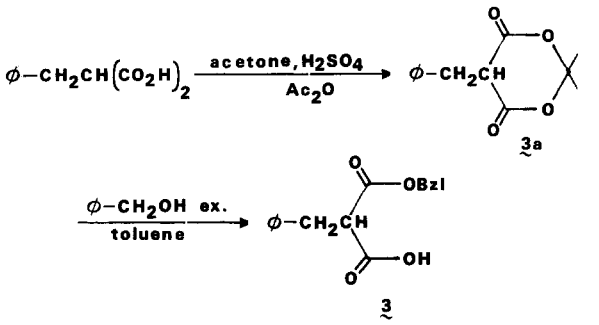
Results

Scheme I outlines the synthetic route followed for the synthesis of [gpGlu^6,(*RS*)-mPhe^7]SP$_{6-11}$ (II). The rearrangement of *N*-(benzyloxycarbonyl)-L-pyroglutamic acid (1) to the *N*,*N*′-bis(benzyloxycarbonyl)-5-aminopyrolidin-2-one (2) was accomplished by a multistep procedure in which intermediates were used after partial purification. The mixed anhydride (**1a**) formed upon reaction of the N-protected amino acid with ethyl chloroformate was converted in situ to the appropriate acyl azide (**1b**) by treatment with a large excess of an aqueous solution of sodium azide, following a procedure of Sheehan et al.[23]

Curtius rearrangement of the acyl azide intermediate (**1b**) was achieved in toluene at 80 °C. Progress of the reaction was monitored by infrared analysis of aliquots of the reaction mixture. Disappearance of the acyl azide band at 2150 cm^{-1}, with a concomitant appearance of the isocyanate band at 2250 cm^{-1}, indicated the completion of the rearrangement. Once the rearrangement was accomplished, the isocyanate intermediate (**1c**) was trapped by a large excess of benzyl alcohol, thus yielding the N-protected *gem*-diamino residue **2**, derived from the pyroglutamic acid derivative **1**.

Deprotection of **2** via catalytic heterogeneous hydrogenation yielded the free 5-aminopyrrolidin-2-one (**2a**), which was immediately coupled to benzyl hydrogen (*RS*)-2-benzylmalonic acid (**3**). This coupling, which yielded the pseudodipeptide unit **4**, was accomplished with DCC and HOBt as an additive.[24]

Scheme II. Synthesis of Benzyl Hydrogen (*RS*)-2-Benzylmalonate (**3**)



Removal of the carboxy protecting group through catalytic hydrogenation yielded the free *N*-(2-benzylmalonyl)-5-aminopyrrolidin-2-one (**4a**). The synthesis of II was accomplished after coupling of **4a** with the free tetrapeptide amide **5a**, under conditions similar to those employed for the synthesis of **4**. The crude peptide II was purified by column chromatography on silica gel.

For the preparation of the monoester of the 2-substituted malonic acid **3**, we followed a synthesis outlined in Scheme II. The formation of 2,2-dimethyl-5-benzyl-1,3-dioxane-4,6-dione (**3a**) (Meldrum's acid derivative[25]), from 2-benzylmalonic acid, was followed by ring opening with a large excess of benzyl alcohol to yield the monoester **3**. This route has some advantage over the alternative procedure, which involves partial saponification of malonic acid diester.

Scheme III outlines the synthetic steps employed for the preparation of PMRI analogue III. The protected dipeptide hydrazide (**7**) was chosen as the starting material for the Curtius rearrangement. A procedure reported by Honzl and Rudinger[26] was employed to transform the hydrazide **7** into the corresponding acyl azide (**7a**). The course of the rearrangement of **7a** was monitored by infrared spectroscopy as in the preparation of **1c**. Quenching of the isocyanate intermediate **7b** was achieved with a large excess of dry malonic acid, which furnished directly the N-protected carboxy-free pseudotripeptide unit **8**. The reaction between an isocyanate and a carboxylic acid was studied and used by Goldschmidt and Wick for the coupling of *N*-carbonyl amino acid ester with an N-protected amino acid.[27]

The N-protected pseudotripeptidic fragment **8** was coupled via *N*,*N*′-dicyclohexylcarbodiimide (DCC) in the presence of 1-hydroxybenzotriazole (HOBt) to the dipeptide amide **5b**. Acidolytic deprotection of the pseudopentapeptide **9** gave the corresponding free pseudopentatpeptide **9a**, which was then coupled to pyroglutamic acid by the "mixed anhydride" method, yielding the PMRI analogue III. The crude product was purified on silica gel.

In the two procedures outlined in Schemes I and III, the strategy of synthesis was based on the immediate incorporation of a *gem*-diamino residue into the peptide backbone, e.g., **4** and **8**. Such an approach minimizes the need for further synthetic manipulations that involve a *gem*-diamino residue with a free amino function. A free *gem*-

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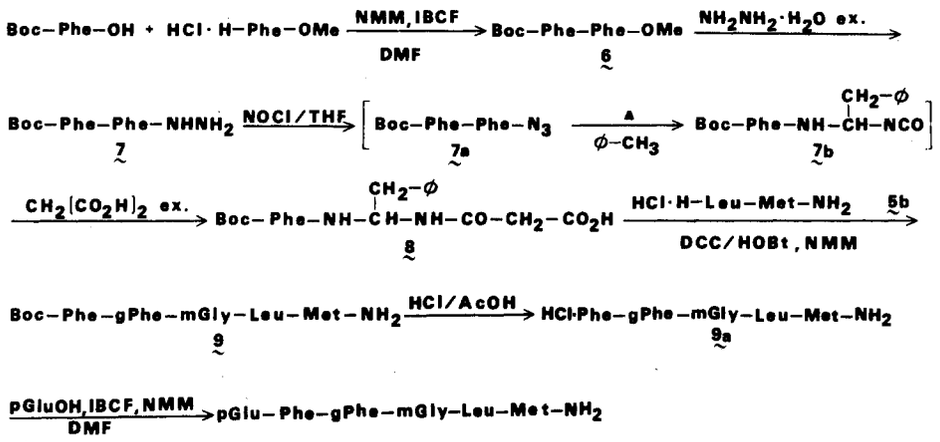
Scheme III. Synthesis of [pGlu^6,gPhe^8,mGly^9]SP$_{6-11}$ (III)



III

Table I. Analytical and Chromatographic Characterization and Biological Activity of PMRI Analogues of [pGlu^6]SP$_{6-11}$

	PMRI analogues of [pGlu^6]SP$_{6-11}$ (I)	
	[gpGlu^6,(*RS*)-mPhe^7]SP$_{6-11}$ (II)	[pGlu^6,gPhe^8,mGly^9]SP$_{6-11}$ (III)
	Analytical Characterization	
elemental anal.	($C_{36}H_{49}N_7O_7S$) C, H, N	($C_{36}H_{49}N_7O_7S$) C, H, N
FD mass spectra,[a] (*m/e*)		
MH^+	724	724
MK^+	762	762
amino acid anal.: (calcd) found		
Glu	(–) –[b]	(1.00) 1.08
Phe	(1.00) 1.02[c]	(1.00) 1.00[b]
Gly	(1.00) 0.95	(–) –[c]
Leu	(1.00) 1.00	(1.00) 1.09
Met	(1.00) 1.03	(1.00) 1.04
NH_3	(3.00) 2.95	(3.00) 2.91
	Chromatographic Characterization	
HPLC		
k' (% H_2O/% MeOH)	5.71 (40/60)	3.38 (50/50)
TLC		
R_f (solv system)	0.52 (A), 0.75 (C), 0.25 (I)	0.15 (A), 0.51 (C), 0.62 (E)
	Biological Activity[d]	
rel potency,[e] %	<0.01	22
ED_{50},[f] nM		0.9

[a] Calcd M_r = 723; see ref 28. [b] A *gem*-diamino residue yields upon acidic hydrolysis 2 equiv of ammonia and the corresponding aldehyde. [c] A malonyl residue is not detectable by amino acid analysis. [d] The in vitro test performed was the contraction of guinea pig ileum according to ref 34. [e] The spasmogenic activity of the parent peptide [pGlu^6]SP$_{6-11}$ (I) was taken as 100% with an ED_{50} of 0.2 nM. [f] Concentration of agonist in the organ bath corresponding to 50% of the maximal measured spasmogenic effect. [g] For composition of solvent system, see Experimental Section.

diamino residue might be subjected to decompositions due to its inherent chemical reactivity. In the case of the synthesis of *N*-malonyl-*N'*-[(*tert*-butyloxycarbonyl)-L-phenylalanyl]-1,1-diamino-2-phenylethane (**8**), this concept is ultimately fulfilled, since the amino functions of the 1,1-diamino-2-phenylethane moiety are acylated throughout the synthesis. The reason for not following the same route in the synthesis of **4a** was due to the poor yield obtained in the attempted trapping of isocyanate **1c** with the 2-substituted malonic acid derivative.

The purity of analogues II and III was extensively examined by TLC, HPLC, amino acid analysis, elemental microchemical analysis, high-resolution ^{1}H NMR, and potassium-cationized field-desorption mass spectrometry (see Table I) This mass spectrometric procedure was recently introduced and found to be very helpful for the elucidation of structure and evaluation of purity of oligopeptides.[28]

The synthesis of peptide sequences **5a**, **5b**, and **6** was based on the "excessive mixed carbonic–carboxylic acid anhydrides" method introduced by Tilak.[29] This method ensures complete acylation of the amino component, which is verified by the negative fluorescamine test.[30] The excess mixed anhydride is hydrolyzed by $KHCO_3$. The *tert*-butyloxycarbonyl group,[31] used for N protection of the α-amino function, was removed by 1 N HCl in glacial acetic acid,[32] or in HCl in ethyl acetate,[33] in the presence of 1% thioanisole.

The detailed procedures for the preparation of **5a** and **5b** and their characterization will be published elsewhere.

The PMRI analogues II and III were subjected to an in vitro test, in which contraction of isolated guinea pig ileum

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was induced by variable concentrations of the analogues.[34] Relative potencies and ED_{50} values were obtained from dose–response curves. The biological results shown in Table I indicate that analogue II, in which the peptide bond pGlu^6-Phe^7 was reversed, is devoid of any spasmogenic activity. On the other hand, the PMRI analogue III, in which the peptide bond Phe^8-Gly^9 was reversed, had a relative potency of 22%. Analogue III is a full agonist of SP, since a maximal spasmogenic activity was observed at sufficiently high doses.

Discussion

The preparation of PMRI analogues of peptides is not yet a routine straightforward synthesis. The major difficulties stem from low reaction yields, which necessitate extensive purifications of intermediates and final products.

The synthesis of analogue II resulted in a mixture of two diastereomers, since the 2-benzylmalonyl residue incorporated into fragment 4 was racemic. At this stage of the synthesis we could determine by HPLC the two diastereomers of the pseudodipeptide 4; however, the lack of biological activity of PMRI analogue II did not justify the labor needed for separation and configurational assignment.[35] The configuration of the *gem*-diamino residues obtained via Curtius rearrangement is retained. The synthesis of PMRI analogue III did not result in a diastereomeric mixture, since the malonyl residue in this analogue was achiral.

The extended structures of the parent peptide I and its pseudopeptide analogues II and III share only topochemical resemblance. We, as well as others, do not assume absolute topochemical identity.[19,36,37] The reversed peptide bond is isoelectronic and isosteric with the original peptide bond, but theoretical studies reveal that the non amino acid residues show conformational preferences, different from those characteristic for amino acid residues.[38] Therefore, we anticipate that the retro-inverso modification of a single peptide bond will result in different folded conformations for the parent peptide and its PMRI analogues.

The effect of a single peptide bond reversal on the biological activity and on conformation may also be attributed to possible alterations in important interactions of this particular peptide bond with a receptor or within the same molecule. The reversal of a peptide bond may perturb molecular interactions, such as hydrogen bonds. In this regard, it is interesting to point out that an N-methylated analogue of I, i.e., [pGlu^6,*N*-MePhe^7]SP_{6-11}, was found to possess substantial reduced biological activity,[18] as was the analogue [gpGlu^6,(*RS*)-mPhe^7]SP_{6-11} (II). Evidently, structural modification of the pGlu^6-Phe^7 peptide bond has a large effect on biological activity. Conformational studies of these analogues, in an attempt to pick up unique features, different from those of the active parent peptide [pGlu^6]SP_{6-11}, are in progress.

The reversal of the peptide bond Phe^8-Gly^9, which results in the incorporation of two non amino acid residues, yielded analogue III, a full agonist with substantial potency. The receptor interacting with PMRI analogue III can accommodate this pseudopeptidic structure with great efficiency. This might suggest that the interaction of the peptide bond Phe^8-Gly^9 with the receptor or its conformational contributions are of minor significance to the biological activity of the PMRI analogue III. Further biological studies, as well as the metabolic stability of these analogues, will be published elsewhere.

Experimental Section

Melting points were taken on a Thomas-Hoover capillary melting point and are uncorrected. IR spectra were recorded on a Perkin-Elmer 457 spectrophotometer. Optical rotations were measured on a Perkin-Elmer 141 with a 10-cm water-jacketed cell.

HPLC analysis was performed on a Tracor 950 liquid chromatograph equipped with Tracor 970A variable-wavelength detector and Tracor 980A solvent programmer. During HPLC analysis, peptides were monitored at two wavelengths, 210 and 254 nm. An Altech C-18, 10-μm column (4.6 mm i.d. × 25 cm) was used in the HPLC system. TLC was run on precoated silica gel plastic plates: Polygram Sil NH-R/UV254 purchased from Macherey-Nagel Co. The following solvent systems were used: (A) $CHCl_3$/MeOH (9:1), (B) $CHCl_3$/MeOH (1:1), (C) $CHCl_3$/MeOH (4:1), (D) $CHCl_3$/MeOH/AcOH (20:20:1), (E) $CHCl_3$/MeOH/AcOH (17:2:1), (F) EtOAc/pyridine/AcOH/H_2O (5:5:1:3), (G) EtOAc/hexane (1:1), (H) EtOAc/MeOH (1:1), (I) $CHCl_3$/MeOH (19:1). The plates were developed with spray reagents, ninhydrin 0.1% (Merck) or fluorescamine (Fluram, Hoffman La Roche & Co. AG) and/or with iodine vapors. Amino acid analysis was obtained on LKB-4400 amino acid analyzer equipped with a Spectra-Physics SP-4100 printer–plotter computing integrator using 4-component sodium buffer systems and a standard 70-min program. Hydrolysis of peptide samples for amino acid analysis was carried out on 1-mg samples in constant-boiling HCl (0.5 mL), which was degassed, sealed under high vacuum, and heated at 110 °C for 20 h. The hydrolysate was dried under vacuum over KOH pellets and diluted with 0.2 N sodium citrate buffer (0.2 mL), pH 2.2.

Elemental microchemical analysis was carried out at the Microanalytical Laboratory of the Organic Chemistry Department. Where elemental analyses are indicated only by symbols of the elements, analytical results were within ±0.3% of the theoretical values.

Potassium-cationized field-desorption mass spectrometry was performed on a Varian CH5-DF mass spectrometer equipped with an EI, FI, and FD ion source (for more details, see ref 28). Column chromatography was used extensively for preparative purification of crude products. Open column chromatography was run on silica gel 60 (70–230 mesh, Merck). Low-pressure liquid chromatography was run on a Michel-Miller high-performance, low-pressure liquid chromatography (HPLPLC) column system (ACE glass inc.) using a Fluid Metering Inc. lab pump RP-SY-2-CSC and silica gel 60 (230–400 mesh, Merck). The effluent was monitored by an ISCO-UA5 multiwavelength absorbance monitor, at 254 nm.

Solvent Purification. $CHCl_3$ was distilled from P_2O_5; dimethylformamide (DMF) and hexamethylphosphoramide (HMPA) were distilled from P_2O_5 under reduced pressure. DMF was then redistilled from ninhydrin. Acetic acid was distilled from $B(OAc)_4$. THF, toluene, and benzyl alcohol were distilled from CaH_2 under nitrogen.

Coupling Procedures. A. To a stirred solution of Boc amino acid or Boc peptide (1.3–1.5 equiv) in DMF (0.4 to 0.7 M) an equivalent of *N*-methylmorpholine (NMM) was added. The mixture was cooled to −20 °C and kept under nitrogen. Isobutyl chloroformate (IBCF) (90% of the N-protected component) was added and allowed to react for 2 min. A precooled solution of amino component hydrochloride (1.0 equiv) in DMF (0.3 to 0.5 M) was added to the reaction mixture, followed by NMM (1.0 equiv). After completion of the reaction (about 2 h, monitored by Fluram), the temperature was allowed to reach 0 °C, and the reaction mixture was treated with 2 M $KHCO_3$ (3.0 equiv) for 30 min. (a) Addition of saturated aqueous NaCl solution (4 × volume of DMF) resulted in precipitate formation. The precipitate was collected and washed with distilled water, until washings were free of Cl^-. The precipitate was dried under vacuum over P_2O_5.

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(b) If a precipitate did not form or the precipitate yield was low after addition of water to the reaction mixture, the solvents were removed under reduced pressure. The residue was redissolved in ethyl acetate and washed consecutively with brine, 0.5 N $KHSO_4$, brine, 5% $NaHCO_3$, and brine. The organic phase was dried over $MgSO_4$, filtered, and taken to dryness under reduced pressure. The residue obtained was further purified by column chromatography.

B. A solution of the carboxy component (1.0–1.1 equiv), the amino component (1.0 equiv), NMM (1.0 equiv, only in case where the amino component was introduced as the HCl salt), and HOBt (2.2 equiv) in DMF was stirred and cooled to 0 °C. A solution of DCC (1.1 equiv) in DMF was added to the reaction mixture. The final concentration of the amino component was 0.25–0.4 M. The reaction mixture was allowed to reach room temperature and stirred for 12 h. To the cooled reaction mixture (0 °C) were added a few drops of AcOH, and the mixture was allowed to react for 30 min at room temperature. The reaction mixture was cooled again and filtered from the *N,N'*-dicyclohexylurea (DCU); the filtrate was then washed with cold DMF. The combined filtrate and washings were taken to dryness under reduced pressure, and the residue was dissolved in EtOAc and treated according to A,b.

Deprotection. A. Boc-protected peptides (1 g/50 mL) were treated with (a) 1 N HCl in glacial acetic acid[32] or (b) HCl in EtOAc[33] in the presence of 1% thioanisole. The solution was kept for 30 min at room temperature, protected with $CaCl_2$. Removal of solvent under reduced pressure was followed by storing the residue overnight under vacuum, over KOH pellets. The solid was recrystallized from MeOH–ether, collected by filtration, and washed with ether.

B. Catalytic hydrogenation was performed in a Parr apparatus. Benzyloxycarbonyl-protected peptides or benzyl ester was dissolved in the appropriate solvent and flushed with nitrogen. The catalyst, 10% Pd/C, was added (catalyst to peptide ratio, 1:9 w/w, and the hydrogenation was carried out under 50 psi of H_2 for 1 to 2 h. The reaction mixture was filtered through a Celite bed. The filtrate was evaporated under reduced pressure, and the residue was treated as described below.

***N,N'*-Bis(benzyloxycarbonyl)-5-aminopyrrolidin-2-one (2).** *N*-Carbobenzoxy-L-pyroglutamic acid (1, 7 g, 26.6 mmol) was dissolved in THF (50 mL). To the stirred solution at −15 °C under nitrogen were added triethylamine (TEA; 3.68 mL, 26.6 mmol) and ethyl chloroformate (2.5 mL, 26.6 mmol). After 10 min, the reaction mixture was transferred to an ice bath and an aqueous solution of 10 M NaN_3 (30 mL) was added and allowed to react for 30 min. The reaction mixture was extracted with cold EtOAc (3 × 150 mL). The combined EtOAc extracts were washed with brine (3 × 150 mL) and dried over $MgSO_4$, at 0 °C. The solvent was removed under reduced pressure to give the acyl azide **1b** (IR 2150 cm^{-1}), which was redissolved in toluene (100 mL). The stirred solution of the acyl azide **1b** was heated at 80 °C, under N_2, until complete conversion (~5 min) to the corresponding isocyanate **1c** occurred (IR 2250 cm^{-1}). Benzyl alcohol (4.2 mL, 40 mmol) was added to the isocyanate solution, and the mixture was stirred under N_2 for 30 min at 80 °C and for 12 h at 60 °C. The reaction mixture was cooled, and the precipitate collected by filtration, washed with toluene, and dried under vacuum over paraffin oil: yield 6.9 g (70%); TLC R_f (A) 0.58, R_f (H) 0.62, R_f (I) 0.52; mp 147–150 °C; $[\alpha]^{25}_D$ −5.5° (*c* 1.0, MeOH). Anal. ($C_{20}H_{20}N_2O_5$) C, H, N.

2,2-Dimethyl-5-benzyl-1,3-dioxane-4,6-dione (3a). To a stirred and cooled (0 °C) suspension of 2-benzylmalonic acid (20 g, 0.10 mol) in acetic anhydride (50 mL) was added dropwise concentrated H_2SO_4 (2 mL). This was followed by the dropwise addition of dry acetone (12.5 mL, 0.17 mol), over a period of 10 min. The reaction mixture was allowed to react at room temperature for 1 h and then cooled in an ice bath. Crushed ice (50 g) was added to the reaction with vigorous stirring. The precipitate was collected by filtration, washed thoroughly with cold water, and dried over P_2O_5 in vacuo. The crude product was recrystallized from MeOH–heptane: yield 10.5 g (45%); TLC R_f (A) 0.70, R_f (G) 0.38; mp 78–79 °C. Anal. ($C_{13}H_{14}O_4$) C, H.

Benzyl Hydrogen (*RS*)-2-Benzylmalonate (3). To a stirred solution of **3a** (9 g, 38.5 mmol) in toluene (7 mL) was added benzyl alcohol (8 mL, 77 mmol), and the reaction mixture was kept under nitrogen at 70 °C for 12 h. Removal of solvent under reduced pressure gave an oily residue, which was subjected to open column chromatography (2.0 × 50 cm). The eluent was a gradient of EtOAc in hexane (0 to 10%). Removal of the solvent gave the oily monobenzyl ester **3**: yield 9.5 g (90%); TLC R_f (A) 0.24, R_f (E) 0.60. Anal. ($C_{17}H_{16}O_4$) C, H.

gpGlu-(*RS*)-mPhe-OBzl (4). A suspension of **2** (5 g, 13.3 mmol) in EtOAc was deprotected according to general procedure B for 1.5 h. The oily residue (**2a**) obtained following removal of the solvent under reduced pressure was redissolved in DMF (6 mL). To this solution was added the monoester **3** (4.1 g, 14.6 mmol in DMF), followed by HOBt (4 g, 29.3 mmol) and DCC (3 g, 14.6 mmol). Coupling followed general procedure B. The crude product **4** (4.5 g) was subjected to open column chromatography (2.5 × 100 cm). For elution, a gradient of MeOH in $CHCl_3$ (0 to 3%) was employed, followed by low-pressure liquid chromatography (5 × 50 cm) with a gradient of MeOH in $CHCl_3$ (0 to 5%). The pseudodipeptide **4**, yield 1.8 g (30%), was pure in HPLC (H_2O/MeOH, 40:60), k_1' = 3.11 and k_2' = 3.38 (50:50), k_1' = 16.9 and k_2' = 18.6 (k_1' and k_2' correspond to the two diastereoisomers composing the mixture): TLC (R_f (A) 0.54, R_f (I) 0.30; mass spectrum, *m/e* 366 (M^+). Anal. ($C_{21}H_{22}N_2O_4$) C, H, N.

[gpGlu^6,(*RS*)-mPhe^7]SP$_{6-11}$ (II). (a) Deprotection of **4** (1.5 g, 4.1 mmol) in EtOAc (75 mL) according to general procedure B took 20 h. The solid **4a**, obtained by removal of EtOAc, was redissolved in DMF (14 mL).

(b) To the above solution of **4a** in DMF was added the tetrapeptide hydrochloride **5a** (2.1 g, 4.1 mmol), followed by NMM (0.46 mL, 4.1 mmol) and HOBt (1.2 g, 8.8 mmol). The mixture was cooled in an ice bath and stirred under nitrogen. DCC (0.95 g, 4.5 mmol) dissolved in DMF (3 mL) was added to the reaction mixture. Coupling was according to general procedure B. The crude product was purified by two consecutive silica gel columns (5 × 50 cm followed by 2.5 × 100 cm) employing the low-pressure liquid chromatography technique. A gradient of MeOH in $CHCl_3$ (0 to 5%) was used to elute analogue II. Evaporation of the solvent from the appropriate combined fractions gave solid II, which was recrystallized from MeOH–ether, yielding 580 mg (20%) of pure II. Full characterization of the analogue II is summarized in Table I.

Boc-Phe-Phe-OMe (6). Coupling was performed according to general procedure A. Boc-Phe-OH (4.5 g, 16.8 mmol) was reacted with IBCF (2.1 mL, 16.1 mmol) in the presence of NMM (1.9 mL, 16.8 mmol) in DMF (8.5 mL). The amino component HCl·H-Phe-OMe (3.09 g, 14.1 mmol) was dissolved in DMF (8.5 mL) in the presence of NMM (1.6 mL, 14.1 mmol). The protected dipeptide was recrystallized from EtOAc–hexane: yield 5.5 g (91.5%); mp 117–119 °C; $[\alpha]^{25}_D$ −11.7° (*c* 1.0, MeOH) [lit.[39] mp 121–122 °C; $[\alpha]_D$ −14.2° (*c* 1.0, MeOH)]; TLC R_f (G) 0.43; R_f (I) 0.63. Anal. ($C_{24}H_{30}N_2O_5$) C, H, N.

Boc-Phe-Phe-NH-NH$_2$ (7). To an ice-cold stirred solution of **6** (4. g, 9.4 mmol) in DMF (3 mL) was added hydrazine hydrate 99% (9.1 mL, 18.8 mmol). The reaction mixture was stirred at room temperature for 12 h. The precipitate was collected by filtration, washed with ether, and dried over H_2SO_4 (conc) in vacuo. The crude hydrazide **7** was recrystallized from EtOH: yield 2.4 g (60%); mp 180–181 °C; $[\alpha]^{25}_D$ −18.5° (*c* 1.0, DMF) [lit.[39] mp 179–180 °C; $[\alpha]_D$ −22.5° (*c* 1.0, DMF)]; TLC R_f (A) 0.52; R_f (C) 0.72; R_f (H) 0.67. Anal. ($C_{23}H_{30}N_4O_4$) C, H, N.

Boc-Phe-gPhe-mGly-OH (8). (a) To a stirred suspension of **7** (2.5 g, 5.8 mmol) in dry THF (80 mL) at 0 °C was added a precooled saturated solution of NOCl in dry THF until complete dissolution of the hydrazide. The reaction mixture was diluted with ice-cold EtOAc (400 mL) and washed with a cold solution of 5% $NaHCO_3$ (50 mL), followed by cold brine. The EtOAc solution was dried over $MgSO_4$ in an ice bath and taken to dryness to yield the acyl azide **7a** (IR 2150 cm^{-1}).

(b) The acyl azide **7a** was suspended in dry toluene (25 mL) and heated at 80 °C under nitrogen. The completion of rearrangement (10 min) was determined by the appearance of an IR band at 2250 cm^{-1} corresponding to the isocyanate **7b** and the disappearance of the band corresponding to the acyl azide.

(c) A large excess of malonic acid (5 g, 29 mmol) was added to the solution of the isocyanate **7b** in toluene heated at 80 °C.

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Reaction took place with strong liberation of gas and was completed after 3 min (disappearance of IR band at 2250 cm^{-1}). The precipitate from the cold reaction mixture was collected by filtration and washed with toluene. The dry solid was washed with water and dried over P_2O_5 in vacuo to give compound 8: yield 1.9 g (70%); mp 182–185 °C dec; $[\alpha]^{25}_D$ −1.9° (c 1, DMF); TLC R_f (B) 0.53, R_f (E) 0.62. Anal. ($C_{25}H_{31}N_3O_6$) C, H, N.

Boc-Phe-gPhe-mGly-Leu-Met-NH_2 (9). To a solution of 8 (1.7 g, 3.6 mmol) in DMF (15 mL) were added HOBt (1.06 g, 7.8 mmol), NMM (0.4 mL, 3.6 mmol), and the amino component **5b** (1.1 g, 3.6 mmol). A solution of DCC (0.82 g, 3.9 mmol) in DMF (5 mL) was cooled and added to the stirred reaction mixture kept in ice bath. The coupling procedure and the workup followed general procedure B. The crude product was loaded on a silica gel column (5 × 25 cm, 70–230 mesh) and eluted by a gradient of MeOH in $CHCl_3$ (5 to 10%). The product obtained from chromatography was recrystallized from MeOH–ether. The precipitate was collected by filtration, and the white solid 9 was dried over P_2O_5 in vacuo: yield 0.95 g (35%); $[\alpha]^{25}_D$ −13.4° (c 1.0, DMF); mp 226–228 °C; TLC R_f (A) 0.32; R_f (E) 0.64, R_f (H) 0.67; HPLC (H_2O–MeOH, 25:75 and 30:70) k' = 3.87, k' = 8.75, respectively. Anal. ($C_{36}H_{52}N_6O_7S$) C, H, N. Amino acid analysis calcd for Phe/Leu/Met/NH_3, 1:1:1:3; found, 0.93:1:1:2.80.

HCl·H-Phe-gPhe-mGly-Leu-Met-NH_2 (9a). Compound 9 (0.45 g, 0.62 mmol) was deprotected according to general procedure A. The crude product was recrystallized from MeOH–ether to give **9a** as a solid: yield 0.31 g (77%); $[\alpha]^{25}_D$ −3.0° (c 0.3, AcOH); mp 223–225 °C dec; TLC R_f (B) 0.59; R_f (D) 0.64; R_f (E) 0.52. Anal. ($C_{31}H_{45}N_6O_5SCl$) C, H, N.

pGlu-Phe-gPhe-mGly-Leu-Met-NH_2 (III). The coupling and workup followed general procedure A. To a solution of pGlu-OH (0.166 g, 1.29 mmol) in DMF (2.5 mL) was added NMM (0.144 mL, 1.29 mmol). After cooling of the reaction mixture, IBCF (0.15 mL, 1.2 mmol) was added. A solution of the amino component **9a** (0.26 g, 0.4 mmol) in HMPA (3 mL) was treated with NMM (0.045 mL, 0.4 mmol), cooled, and added to the reaction mixture. The analogue III was obtained from the reaction mixture as a white powder: yield 0.26 g (90%); $[\alpha]^{25}_D$ −24° (c 0.1, dimethyl sulfoxide); mp 230–231 °C (full characterization of analogue III is summarized in Table I).

In Vitro Bioassay. Guinea Pig Ileum Assay. The activity of analogues I–III was assayed on an isolated guinea pig ileum as described by Rossel et al.[34] with the following modifications. The ileum was suspended in a 10-mL organ bath containing Tyrode solution (118 mM NaCl, 4.7 mM KCl, 1.8 mM $CaCl_2$, 0.5 mM $MgSO_4$, 0.5 mM NaH_2PO_4, 25.0 mM $NaHCO_3$, 11 mM D-glucose), thermostated at 32 °C, and bubbled with 95% O_2 and 5% CO_2. The segment of the ileum was left to equilibrate for 30 min, before introduction of the tested compounds, and washed every 10 min with Tyrode solution. Isotonic contractions of the longitudinal muscles were measured with a smooth-muscle transducer purchased from Harvard Apparatus and recorded on a chart mover. We prepared fresh stock solutions of the peptides (10^{-3} M) by dissolving the peptides in Me_2SO. Further dilutions (up to 10^{-9} M) were performed with Me_2SO. The final concentration of Me_2SO in the organ bath did not exceed 0.1% (v/v).

Acknowledgment. We acknowledge the National Institutes of Health (Grant NS16658-02) for their generous support of this study. The authors also express their gratitude to Dr. S. Blum and her staff for the microanalytical service.

Registry No. 1, 32159-21-0; **1a**, 82379-40-6; **1b**, 82379-41-7; **1c**, 66488-77-5; **2**, 66488-70-8; **2a**, 40759-90-8; **3**, 82379-43-9; **3a**, 3709-27-1; **4** (isomer 1), 83815-83-2; **4** (isomer 2), 83803-08-1; **4a**, 82379-45-1; **5a**, 58172-54-6; **5b**, 2131-00-2; **6**, 13122-89-9; **7**, 40099-25-0; **7a**, 82379-46-2; **7b**, 82379-47-3; **8**, 83803-09-2; **9**, 82379-49-5; **9a**, 83860-21-3; II, 79775-20-5; III, 82379-39-3; Boc-Phe-OH, 13734-34-4; H-Phe-OMe·HCl, 7524-50-7; pGlu-OH, 98-79-3; ethyl chloroformate, 541-41-3; 2-benzylmalonic acid, 616-75-1; malonic acid, 141-82-2; acetone, 67-64-1.

Folate Analogues. 20. Synthesis and Antifolate Activity of 1′,2′,3′,4′,5′,6′-Hexahydrohomofolic Acid[1]

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The synthesis of 1′,2′,3′,4′,5′,6′-hexahydrohomofolic acid (3), a close analogue of homofolic acid (2), has been carried out by replacement of the benzene ring of 2 with a cyclohexane ring. The synthetic methods employed here were based on the Boon–Leigh strategy to obtain products with unambiguous structures. Based on a number of chemical and spectral observations, a tentative cis stereochemistry was assigned to the 1,4-substituents of the cyclohexane ring of both the homopteroate analogue 13 and the target compound 3. We investigated hexahydrohomopteroic acid (13), hexahydrohomofolic acid (3), and their 7,8-dihydro and *d,l*-5,6,7,8-tetrahydro derivatives for antifolate activities employing several biological test systems. The dihydro and tetrahydro derivatives of both 13 and 3 were active against *Streptococcus faecium*, whereas they were inactive against *Lactobacillus casei*. These compounds were neither substrates nor inhibitors of *L. casei* dihydrofolate reductase or thymidylate synthase.

Thymidylate synthase (EC 2.1.1.45) catalyzes the terminal step in the de novo synthesis of thymidylic acid, which is required exclusively for DNA synthesis. Because of the unique feature of this enzyme, it continues to be a prime target for cancer chemotherapy. Although potent inhibitors of thymidylate synthase (TS) of the substrate class, such as 5-FUdR, are well known[2] and are being used in the clinical treatment of various human cancers, such inhibitors belonging to the coenzyme class [analogues of the vitamin folic acid (1, Chart I)] are relatively rare. The tetrahydro derivative of homofolic acid (2) synthesized by DeGraw[3,4] was the first known example of a thymidylate synthase inhibitor of the coenzyme class. Goodman and

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0022-2623/83/1826-0135$01.50/0