contained 20 mM Tris-HCl (pH 7.5), 20 mM ATP, 7.5 mM magnesium acetate, 25 mg/mL phosphatidylserine, 0.1 mM EGTA, 0.1 mM $CaCl_2$, 1 nM to 10 μM lyngbyatoxin A analogues, and 1 to 3 mg of enzyme preparation in a final volume of 0.1 mL. The reaction mixture was incubated at 28 °C for 5 min and stopped by spotting onto Whatman p81 filter papers. Under these conditions the assay was linear with time and amount of protein. The filters were washed four times in 75 mM phosphoric acid, dried, and counted in a liquid scintillation counter. Calcium and phospholipid-dependent protein kinase C activity was determined by subtracting the activity determined in the absence of phosphatidylserine and DAG from that in the presence of phosphatidylserine and DAG. In the presence of either Ca^{2+} or phospholipid alone the enzyme activity was less than 5% of the activity when both were present. Protein was determined by the method of Bradford[31] with bovine serum albumin used as a standard.

One unit of protein kinase C activity is defined as that amount of enzyme which catalyzes the transfer of 1 pmol of phosphate from ATP to histone per minute at 28 °C.

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Pseudopeptide Analogues of Substance P and Leucine Enkephalinamide Containing the Ψ(CH_2O) Modification: Synthesis and Biological Activity

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The isosteric methyleneoxy Ψ(CH_2O) function was employed as a novel peptide-bond surrogate and incorporated into sequences of two neuropeptides, substance P (SP) and enkephalin. A pseudopeptide analogue [pGlu^6,Phe^{8}Ψ(CH_2O)Gly^9]SP_{6-11} (7) of SP related C-terminal hexapeptide [pGlu^6]SP_{6-11} and two pseudopeptide analogues of [Leu^5]enkephalinamide, [Tyr^{1}Ψ(CH_2O)Gly^2,Leu^5]enkephalinamide (11) and [Gly^{2}Ψ(CH_2O)-Gly^3,Leu^5]enkephalinamide (17), were synthesized. The N^{α}-protected pseudodipeptidic units were incorporated in the appropriate peptide sequences by using conventional coupling methods in solution. Compound 7 was a potent agonist (EC_{50} = 4.8 nM) of substance P as compared to the parent peptide [pGlu^6]SP_{6-11} (EC_{50} = 1.2 nM), in stimulating contraction of the isolated guinea pig ileum (GPI). Analogue 7 was more potent on the neuronal (NK-3) than on the muscular (NK-1) tachykinin receptors in the GPI as shown by the ratio of activities, EC_{50}(NK-1)/EC_{50}(NK-3) = 3.16, thus displaying an improved selectivity for the NK-3 tachykinin receptor subtype as compared to that of [pGlu^6]SP_{6-11}, EC_{50}(NK-1)/EC_{50}(NK-3) = 0.44. In the rat vas deferens (RVD) assay, a typical NK-2 system, the pseudopeptide analogue 7 was (EC_{50} = 2 μM) 10-fold more potent than the parent peptide and 20-fold less potent than eledoisin, an NK-2 selective tachykinin. The pseudopeptide enkephalin analogue 17 had low biological activity when tested in the electrically induced GPI (EC_{50} = 2.3 μM) and was inactive in the mouse vas deferens (MVD) assay. In the rat brain membrane (RBM) binding assay analogue 17 had low affinity (in the micromolar range) for both the μ and δ binding sites. In contrast, analogue 11 was a potent enkephalin agonist (EC_{50} = 30 nM), being equipotent to [D-Ala^2,Leu^5]enkephalinamide (DALE) in the GPI assay. In the MVD, analogue 11 showed a substantially reduced activity (EC_{50} = 92 nM), being about 10-fold less potent than DALE. In the RBM binding assay analogue 11 showed high affinity (in the nanomolar range) for both the μ and δ binding sites with increased selectivity for the δ sites as shown by the ratio of the apparent affinities for both receptors, $K_i(\delta)/K_i(\mu)$ = 2.1. The contribution of the modified peptide bonds in the mode of interaction of SP and enkephalin at their corresponding receptors is discussed.

Backbone modifications of peptide hormones and neuropeptides play an important role in structure-activity relationship studies and were found to affect potency, enzymatic stability, solubility, and conformational properties. Peptide backbone modifications have been introduced in an increasing number of biologically active peptides.[1] Modifications such as Ψ(NHCO)[2-4] Ψ(CONMe),[5] Ψ($COCH_2$),[6,7] Ψ(CSNH),[8] Ψ(CH_2S),[9] Ψ(CH_2NH), Ψ-(CH_2CH_2),[10] and *E* or *Z* Ψ(CH=CH)[11,12] have resulted in several analogues with increased biological activity and

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receptor selectivity. One goal of some of these peptide-bond surrogates, widely differing from each other in their steric, geometric, electronic distribution, and lipophylic properties, is to achieve maximal topographical resemblance with the amide bond in its trans configuration.

In this regard a close approximation in geometrical terms has been obtained with the rigid Ψ((*E*)-CH=CH) isosteric modification.[11] The introduction of amide-bond replacements, at selected sites in the peptide sequence, can function as conformational probes by allowing variable degrees of rotational freedom in the backbone. These modifications may lead to changes in the patterns of intramolecular as well as peptide–receptor hydrogen-bond formation. Moreover, such modifications can lead to local as well as global changes in dipole moments. Potential advantages resulting from backbone modifications can be associated with the enhancement of metabolic stability, improved selectivity toward receptor subtypes, changes from agonistic to antagonistic biological activities, as well as improved pharmacokinetic properties of the peptides such as increased oral bioavailability, better transportability across the blood–brain barrier into the central nervous system, and prolonged duration of action at target tissues. All these make the pseudopeptide-backbone modification in combination with other structural modifications attractive tools, toward the transformation of biologically active peptides into nonpeptidic peptidomimetic structures.[1,13] In our on-going efforts aimed at the stepwise transformation of biologically active peptides into nonpeptidic peptidomimetic structures[14-17] we have recently reported the synthesis of a series of fully protected pseudodipeptidic units containing the methyleneoxy (CH_2O) modification.[18] This novel modification offers a polar, flexible, and proteolytically resistant surrogate to the amide bond. We have also speculated that the CH_2O function is better corresponding, in geometric terms, to the amide bond and presents a more desirable surrogate to the peptide bond, as compared to the closely related methylenethio (CH_2S)[9] function. The methylenethio peptide-bond surrogate is nucleophilic, prone to oxidation, and has reduced hydrogen-bond-forming capacity. More recently, the synthesis of highly potent, enzymatically stable inhibitors of renin, containing the methyleneoxy function replacing a peptide bond in the renin inhibitory peptide (RIP) sequence, were reported.[19] In this work we report the synthesis and the biological activities of a pseudopeptide analogue of the C-terminal sequence of substance P (SP), in which the Phe[8]-Gly[9] peptide bond was replaced by the methyleneoxy function and pseudopeptide analogue of [Leu[5]]enkephalinamide containing the Ψ(CH_2O) function replacing either the Tyr[1]-Gly[2] or the Gly[2]-Gly[3] peptide bonds. Several studies have shown that [pGlu[6]]SP_{6-11}, a C-terminal hexapeptide analogue of SP, is equipotent to the endogenous undecapeptide itself in most bioassays for SP.[14,20] Therefore, this peptide analogue has been employed as the parent compound in several structure–activity studies.[3,5,7,14,21] The methyleneoxy analogue of [pGlu[6]]SP_{6-11}, [pGlu[6],Phe[8]Ψ(CH_2O)Gly[9]]SP_{6-11} (**7**), prepared in this study was tested in the GPI and RVD assays to determine a possible selectivity for either the NK-1, NK-2, or NK-3 receptor subtypes present in these preparations.[22,23] The Ψ(CH_2O) enkephalin analogues [Tyr[1]Ψ(CH_2O)Gly[2],Leu[5]]enkephalinamide (**11**) and [Gly[2]Ψ(CH_2O)Gly[3],Leu[5]]enkephalinamide (**17**) were tested in the electrically induced GPI and MVD assays and in the rat brain membrane binding assay in order to determine their possible selectivity for either the μ or δ opioid receptor.[24]

Results

The incorporation of the methyleneoxy pseudodipeptide units into the sequences of SP and enkephalin was achieved by a combination of fragment condensation followed by stepwise elongation strategy based on the "excessive mixed carbonic carboxylic acid anhydride" method introduced by Tilak.[25] The preparation of the fully protected pseudodipeptides Boc-PheΨ(CH_2O)Gly-OEt (**1**), Z-Tyr(Bzl)Ψ(CH_2O)Gly-OBu[t] (**8**), and Pht=GlyΨ(CH_2O)Gly-OBu[t] (**12**) was described elsewhere.[18] The pseudopeptide pGlu-Phe-PheΨ(CH_2O)Gly-Leu-Met-NH_2, analogue **7**, was synthesized following the synthetic pathway shown in Scheme I. The pseudodipeptide ester **1** was hydrolyzed in NaOH(aq)/dioxane to give the C-terminal deblocked pseudodipeptide **2**, which was coupled via the mixed anhydride method to the dipeptide H-Leu-Met-NH_2, yielding the pure N^{α}-blocked pseudotetrapeptide **3**. Extention of pseudotetrapeptide **3** to the desired pseudohexapeptide analogue **7** was accomplished in a standard manner. Analogue **7** was isolated in a pure form by recrystallization.

Enkephalin analogue TyrΨ(CH_2O)Gly-Gly-Phe-Leu-NH_2 (**11**) was synthesized according to Scheme II. Pseudodipeptide **8** was deprotected by trifluoroacetic acid to yield pseudodipeptide **9**. Compound **9** was then coupled via the mixed anhydride method to H-Gly-Phe-Leu-NH_2 to give the fully protected pseudopentapeptide **10**, which was isolated in a pure form by recrystallization. Pseudopentapeptide **10** was hydrogenated by the catalytic transfer hydrogenation (CTH) method[23] in the presence of ammonium formate to yield the pseudopentapeptide analogue **11** as the acetate salt in a pure form following lyophilization. The synthesis of pseudopeptide analogue Tyr-

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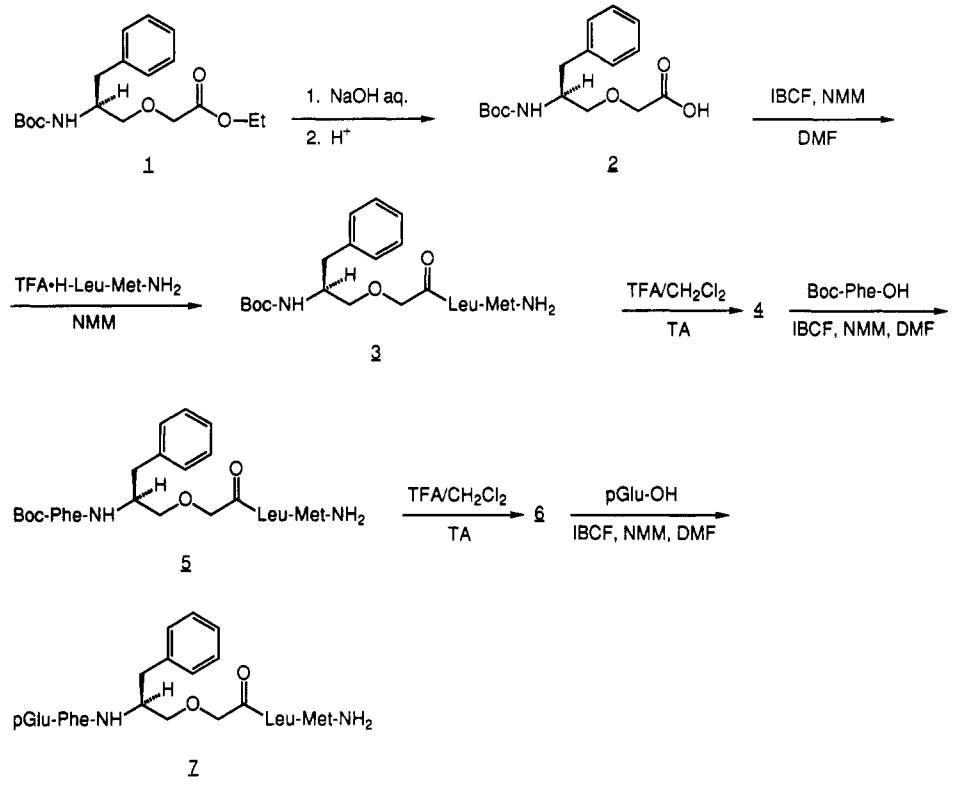
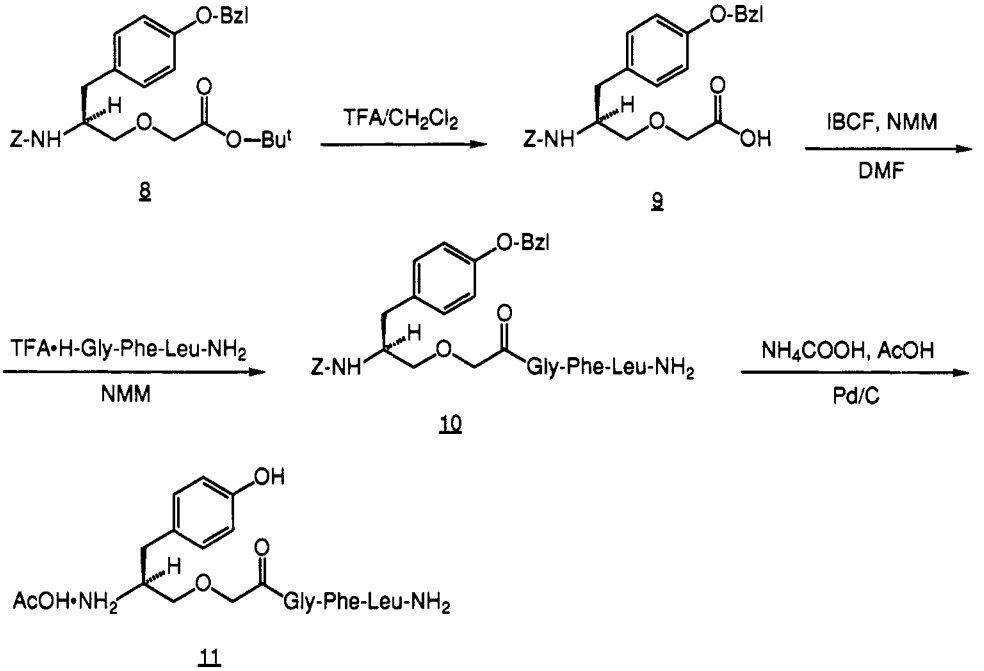
Scheme I. Synthesis of Pseudopeptide Analogue [pGlu^6,Phe$^8\psi$(CH$_2$O)Gly^9]SP$_{6-11}$ (7)



Scheme II. Synthesis of Pseudopeptide Analogue [Tyr$^1\psi$(CH$_2$O)Gly^2,Leu^5]enkephalinamide (11)

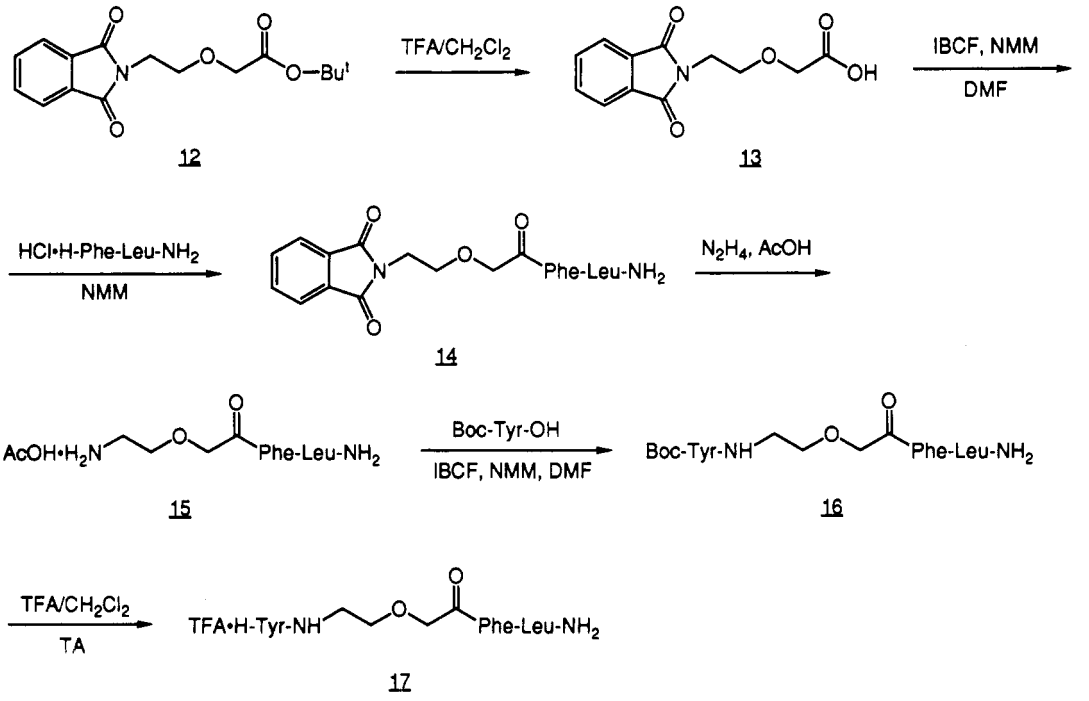


GlyΨ(CH$_2$O)Gly-Phe-Leu-NH$_2$ (17) was carried out according to Scheme III. Removal of the *O-tert*-butyl protecting group from pseudodipeptide 12 was achieved by trifluoroacetic acid to give pseudodipeptide 13. Compound 13 was then coupled by the mixed anhydride method to H-Phe-Leu-NH$_2$ to give N$^\alpha$-protected pseudotetrapeptide 14. Deprotection of Pht-protected pseudotetrapeptide 14 was achieved by N_2H_4/AcOH[24] to yield the pseudotetrapeptide 15 in a pure form as the acetate salt. Compound 15 was then coupled to Boc-Tyr-OH via the mixed anhydride method to give Boc-protected pseudopeptide 16, which was purified by reversed-phase column chromatography. Finally the protected pseudopeptide 16 was deprotected by trifluoroacetic acid to give the desired analogue 17 in a pure form following recrystallization as the trifluoroacetate salt.

The purity of pseudopeptides 7, 11, and 17 as well as that of their intermediates was confirmed by TLC and HPLC. The pseudopeptides were also characterized by elemental analysis, amino acid analysis, and fast atom bombardment (FAB)/tandem mass spectrometry.

The biological activities of SP-related pseudopeptide 7 and of the parent peptide [pGlu^6]SP$_{6-11}$ in the isolated GPI assay and the RVD assay are summarized in Table I. In the isolated GPI assay, analogue 7 [pGlu^6,Phe$^8\Psi$(CH$_2$O)-Gly^9]SP$_{6-11}$ is a potent SP agonist (EC$_{50}$ = 4.8 nM) with

Scheme III. Synthesis of Pseudopeptide Analogue [Gly$^2\psi$(CH$_2$O)Gly^3,Leu^5]enkephalinamide (17)



12 13 14 15 16 17

Table I. Biological Activities of Substance P Analogues and Eledoisin in the Guinea Pig Ileum (GPI) and Rat Vas Deferens (RVD)

compound	GPI EC_{50}, nM NK-1[b]	GPI EC_{50}, nM NK-3[c]	GPI % RP[a]	GPI NK-1/NK-3	RVD: EC_{50}, μM
[pGlu^6]SP$_{6-11}$	1.2 ± 0.3	2.2 ± 0.2	5.0 ± 0.4	100	0.44	20 ± 2.3
[pGlu^6,Phe$^8\psi$(CH$_2$O)Gly^9]SP$_{6-11}$ (7)	4.8 ± 0.5	39.6 ± 3.4	12.5 ± 1.6	40	3.16	2 ± 0.11
[pGlu^6,NMePhe^8]SP$_{6-11}$	0.4 ± 0.01	600 ± 50	0.9 ± 0.04	555	670	20 ± 1.2
eledoisin	3.4 ± 0.6	5.9 ± 0.45	12 ± 0.5	42	0.49	0.1 ± 0.015

[a] RP stands for relative potency, i.e., the ratio of the EC_{50} values of analogue to reference compound [pGlu^6]SP$_{6-11}$ in NK-3 where the latter is taken to be 100%. [b] EC_{50} values were determined in the presence of 3×10^{-7} M atropine. [c] In the presence of 10^{-7} M substance P methyl ester, 2-min preincubation.

Table II. Inhibitory Potencies of Enkephalin Analogues and Morphine in the Guinea Pig Ileum (GPI) and Mouse Vas Deferens (MVD) Assays

compound	GPI IC_{50}, nM	GPI % rel potency	MVD IC_{50}, nM	MVD % rel potency	MVD/GPI
H-Tyrψ(CH$_2$O)Gly-Gly-Phe-Leu-NH$_2$ (11)	30 ± 1.2	1.06	92 ± 2.3	0.13	3.12
H-Tyr-Glyψ(CH$_2$O)Gly-Phe-Leu-NH$_2$ (17)	2300 ± 200	0.014	*c*	*c*	*c*
[D-Ala^2,Leu^5]enkephalinamide	32 ± 2.6	1.0	11.8 ± 0.6	1.0	0.37
[D-Ala^2,Met^5]enkephalinamide	29 ± 0.9	1.1	4.2 ± 0.1	2.8	0.14
H-Tyr-D-Ala-Gly-NMePhe-Gly-ol (DAGO)	18 ± 0.2	1.8	51.5 ± 2.1	0.23	2.84
[D-Ser^2,Leu^5,Thr^6]enkephalin (DSLET)	350 ± 42	0.09	0.54 ± 0.1	21.8	0.0015
[D-Ala^2,D-Leu^5]enkephalin (DADLE)	38 ± 4.1	0.84	0.76 ± 0.2	15.5	0.02
morphine	140 ± 12	0.22	1722 ± 150	0.0068	12.5

[a] Relative potency to [D-Ala^2,Leu^5]enkaphalinamide. [b] Inhibitory activities were reversed by naloxone 10^{-8}–10^{-9} M. [c] Did not show agonist activity up to 10^{-5} M.

25% of the potency of the parent compound [pGlu^6]SP$_{6-11}$. Previous studies have shown that the potencies of tachykinin agonists in stimulating the muscular receptors of the GPI, which have been classified as NK-1 receptors,[26] can be determined in the presence of the muscarinic blocker atropine.[22,23,26,27] On the other hand the activities on the tachykinin neuronal receptors, NK-3 receptors on the guinea pig ileum,[23] were determined following desensitization of the muscular receptors by pretreatment of the smooth muscle with the selective NK-1 agonist substance P methyl ester.[23,27]

As shown in Table I, analogue 7 was about 2-fold less potent than [pGlu^6]SP$_{6-11}$ on the NK-3 receptor whereas it was about 20-fold less potent than [pGlu^6]SP$_{6-11}$ in stimulating the muscular receptor. In the GPI addition of atropine (3×10^{-7} M) followed by desensitization with substance P methyl ester (10^{-7} M) caused a shift to the right, in the dose–response curve, of more than 2 orders of magnitude (results not shown). These results indicate that analogue 7 activates both NK-1 and NK-3 receptors specifically as anticipated from a nonselective SP agonist. The apparant selectivity observed for analogue 7 (EC_{50}(NK-1)/EC_{50}(NK-3) = 3.16) is lower when compared to that of a highly potent NK-3 agonist, [pGlu^6,NMePhe^8]-SP$_{6-11}$, previously reported by us.[23] In the electrically stimulated RVD, a typical NK-2 system,[22] analogue 7 is 10-fold more potent than the parent compound [pGlu^6]-

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Table III. Apparent Affinities of Pseudopeptide Enkephalin Analogues on μ and δ Opioid Receptors of Rat Brain[a]

compound	K_i, nM [^{3}H]DAGO	K_i, nM [^{3}H]DSTBULET	$K_i(\delta)/K_i(\mu)$
H-Tyrψ(CH_2O)Gly-Gly-Phe-Leu-NH_2 (11)	3.69 ± 1.6	7.78 ● 0.9	2.12
H-Tyr-Glyψ(CH_2O)Gly-Phe-Leu-NH_2 (17)	1380 ● 110	9200 ± 1000	6.66
H-Tyr-D-Ala-Gly-NMePhe-Gly-ol (DAGO)	3.9 ± 0.8	629 ● 13	161

[a] Crude rat brain membranes (P_2 fraction), 37 °C, Tris HCl buffer 50 mM, pH 7.4. Concentration of [^{3}H]DAGO = 1 nM, K_d = 3.9 nM. Concentration of [^{3}H]DSTBULET = 2 nM, K_d = 2.2 nM.

SP_{6-11} on the NK-2 receptor (Table I). However, analogue 7 is a weak agonist in the RVD assay (EC_{50} = 2.0 μM), being 20-fold less potent than the NK-2 selective tachykinin eledoisin.

The biological activities of the methyleneoxy pseudopeptide analogues [Tyr$^1\Psi$(CH_2O)Gly^2,Leu^5]enkephalinamide (11) and [Gly$^2\Psi$(CH_2O)Gly^3,Leu^5]enkephalinamide (17) in the electrically stimulated GPI and MVD bioassays[24] are presented in Table II. In the GPI assay analogue 11 is a potent agonist (EC_{50} = 30 nM), equipotent with [D-Ala^2,Leu^5]enkephalinamide[28] and only slightly less potent than the μ agonist DAGO, whereas analogue 17 is a weak agonist, about 70-fold less potent (EC_{50} = 2.3 μM) than [D-Ala^2,Leu^5]enkephalinamide. In the MVD assay, analogue 11 is substantially less potent than [D-Ala^2,Leu^5]enkephalinamide and [D-Ala^2,Met^5]enkephalinamide, with 13% and 4.5% of their potency, respectively. It was also considerably less active than the relatively δ-selective agonists Tyr-D-Ser-Gly-Phe-Leu-Thr (DSLET) and Tyr-D-Ala-Gly-Phe-D-Leu (DADLE). Interestingly, a recent report indicates that replacement of D-Ser^2 in DSLET resulted in Tyr-D-Thr-Gly-Phe-Leu-Thr (DTLET), or cyclization to Tyr-D-Pen-Gly-Phe-Pen improved δ selectivity in a very significant way.[29] In this assay pseudopeptide 17 does not show any agonistic activity even when tested at concentrations of 10 μM. The biological activities of analogues 11 and 17 in the GPI assay and in the MVD were naloxone-reversible. The ratio of the IC_{50} values obtained for opiate agonists in the MVD and GPI assays is usually taken as a measure to indicate μ- or δ-receptor selectivity.[24] As shown in Table II pseudopeptide analogue 11 displays an increased selectivity for the μ receptors; it is about 3-fold more potent in the GPI than in the MVD assay. The ratio IC_{50}(MVD)/IC_{50}(GPI) = 3.2 obtained for analogue 11 is about 8-fold higher than that determined for [D-Ala^2,Leu^5]enkephalinamide and similar to the ratio obtained for Tyr-D-Ala-Gly-*N*-MePhe-Gly-ol (DAGO) (IC_{50}(MVD)/IC_{50}(GPI)) = 2.84, a typical μ-receptor agonist.[30] However, this ratio is considerably lower, about 4-fold, than the ratio observed for the classical μ-receptor agonist morphine.[31,32]

The pseudopeptide analogues 11 and 17 were tested for their relative potency to inhibit the specific binding of [^{3}H]DAGO and [^{3}H]DSTBULET ([D-Ser^2(OBu^t),Leu^5]-enkephalyl-Thr^6) to rat brain membrane (Table III). The binding affinities for both enkephalin analogue 11 and 17 showed a good correlation with the in vitro activities observed in the GPI and MVD. [Gly$^2\Psi$(CH_2O)Gly^3,Leu^5]-enkephalinamide (17) was found to have improved selectivity for μ binding sites labeled by [^{3}H]DAGO as shown by the ratio of the apparent affinities $K_i(\delta)/K_i(\mu)$ = 6.6; however its affinity for both the μ and δ sites was weak in the micromolar range. Pseudopeptide analogue [Tyr$^1\Psi$-(CH_2O)Gly^2,Leu^5]enkephalinamide (11) was found to be about 300-fold more potent than analogue 17 and equipotent to the μ-selective agonist DAGO (EC_{50} = 3.69 nM). Although it exhibited high affinities for both the μ and δ sites, analogue 11 was more selective for the μ sites as shown by the ratio of the apparent affinities $K_i(\delta)/K_i(\mu)$ = 2.1; however this selectivity was much lower than that observed for either μ or δ opioid peptides DAGO and DSTBULET ($K_i(\delta)/K_i(\mu)$ = 161 and 0.0075, respectively).

Discussion

Replacement of amide bonds in the peptide backbone by peptide-bond surrogates addresses one aspect of the extensive search for systematic transformation of bioactive peptides into nonpeptidic peptidomimetic structures. Toward this goal, novel synthetically available pseudopeptidic units, to be used as nonpeptidic building blocks, are required. Thus, the methyleneoxy modification presents a relatively simply available modification with advantageous features such as close geometrical resemblance to the amide bond and a relatively retained degree of polarity, which possesses hydrogen-bond~acceptor capacity, combined with metabolic stability as well as chemical inertness toward oxidation and chemical stability under conditions employed in peptide synthesis.[18] For example, the methyleneoxy pseudodipeptidic units were obtained and incorporated in the sequences of substance P and enkephalin by using a wide range of α-amino- and carboxy-protecting groups, such as benzyloxycarbonyl, *tert*-butyloxycarbonyl, phthalimide, *O*-*tert*-butyl, *O*-benzyl, and *O*-ethyl, which could be efficiently removed without affecting the Ψ(CH_2O) function. This, clearly, is not the case with a number of other amide-bond surrogates, where the choice of protecting groups is limited.[1] Moreover, the increased flexibility of the pseudo-methyleneoxy function compared with that of the amide bond can provide a wider range of conformations, thus facilitating peptide–receptor interactions. Another appealing property of the methyleneoxy modification is its configurational stability which lends itself to fragment condensation without risking racemization.

The Ψ(CH_2O) function was incorporated in the SP sequence in a location which is considered a "silent site", namely at the Phe^8-Gly^9 amide bond which was found to be tolerant to numerous structural modifications without affecting biological potency. Thus replacement of the Phe^8-Gly^9 amide bond by a methyleneoxy function in [pGlu^6]SP_{6-11} resulted in a potent tachykinin agonist. Pseudopeptide 7 with EC_{50} = 4.8 nM in the GPI, which is comparable to that of [pGlu^6]SP_{6-11}, is a weak agonist of the NK-2 receptors on the RVD. Nevertheless, 7 is more potent than [pGlu^6]SP_{6-11} in the RVD assay. These results suggest that the amide bond between Phe^8-Gly^9 does not participate in an essential intermolecular hydrogen-bond interaction with NK-1 receptor subtypes. The activity of this analogue can be compared to that of some other

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Table IV. Relative Potencies of Several Pseudopeptide Analogues of [pGlu^6]SP$_{6-11}$, Containing a Modified Phe^8-Gly^9 Bond

compound[a,b]	% rel potency[c]
[pGlu^6,Phe$^8\psi$(CH$_2$O)Gly^9]SP$_{6-11}$	25
[pGlu^6,Phe$^8\psi$((*E*)CH=CH)Gly^9]SP$_{6-11}$	24[d]
[pGlu^6,Phe$^8\psi$(NHCO)Gly^9]SP$_{6-11}$	22[e]
[pGlu^6,Phe$^8\psi$(COCH$_2$)Gly^9]SP$_{6-11}$	70[f]
[pGlu^6,Sar^9]SP$_{6-11}$	80[g]

[a] As determined in the isolated guinea pig ileum assay. [b] The notation (-)Xxx implies a modification of the peptide bond with the function that substitutes the amide bond between the parentheses. [c] Potencies relative to [pGlu^6]SP$_{6-11}$ = 100%, EC_{50} = 2.0 × 10^{-9} M. [d] Reference 10. [e] Reference 3. [f] Reference 6. [g] Reference 26.

Table V. Relative Potencies of Several Pseudopeptide Analogues of [Leu^5]- and [Met^5]enkephalin Containing a Modified Tyr^1-Gly^2 Bond

compound[a,b]	% rel potency
[Tyr$^1\psi$(CH$_2$O)Gly^2,Leu^5]enkephalinamide	106
[Tyr$^1\psi$(CH$_2$S)Gly^2,Leu^5]enkephalin	24.1[c,d]
[Tyr$^1\psi$(CH=CH)Gly^2,Leu^5]enkephalin-OMe	300[e]
[Tyr$^1\psi$(CH$_2$CH$_2$)Gly^2,Met^5]enkephalinamide	3[f]
[Tyr$^1\psi$(CH$_2$NH)Gly^2,Met^5]enkephalinamide	56[f]
[NMeGly^2,Met^5]enkephalin	<2.1[g]

[a] As determined in the electrically induced guinea pig ileum assay. [b] The notation (-)Xxx implies a modification of the peptide bond with the function that substitutes the amide bond between the parentheses. [c] Receptor binding assay: displacement of [^{3}H]-etorphine from rat brain membranes. [d] Reference 8. [e] Reference 10. [f] Reference 9. [g] Reference 1.

pseudopeptide analogues of [pGlu^6]SP$_{6-11}$ in which the Phe^8-Gly^9 bond has been replaced by relatively more rigid nonpeptidal functions such as Ψ((*E*)-CH=CH) and Ψ-(NHCO) (Table IV). Thus, our results provide further support that the peptide bond between Phe^8-Gly^9 in [pGlu^6]SP$_{6-11}$ can be replaced by a large variety of chemical modifications without leading to serious loss of biological activity.

Previous studies have shown that conformational restriction about the Phe^8-Gly^9 peptide bond in [pGlu^6]SP$_{6-11}$ by either N-methylation of Gly^9 [33] or by substitution of Gly^9 by a proline residue[27] gave rise to agonists with increasing potency and selectivity for the NK-1 receptor subtype. On the other hand, increasing the backbone flexibility about the Phe^8-Gly^9 bond by means of the Ψ-(CH$_2$O) function seems to have a beneficial effect in improving the agonist selectivity for the NK-3 receptors. This lead will be further exploited in future design of novel, nonpeptidic analogues of SP which are highly selective for the NK-3 receptor.

Replacement of the Tyr^1-Gly^2 peptide bond of [Leu^5]-enkephalinamide resulted in a potent agonist for the opiate μ receptors. Analogue 11 displays activity comparable to that of [D-Ala^2,Leu^5]enkephalinamide[28] in inhibiting the electrically induced contractions of the GPI, whereas it is a moderately weak agonist in the MVD assay. In the rat brain membrane binding assay 11 has high affinity for μ- and δ-binding sites with some preference for the μ sites. The ability of analogue 11 to discriminate between the μ and δ receptors is much lower than that observed for the μ-selective agonist DAGO, and is more similar to the μ-selective classical opioid morphine. The activity of pseudopeptide 11 is comparable to that of several other pseudopeptide analogues of enkephalin in which the Tyr^1-Gly^2 peptide bond has been replaced by other pseudopeptidic functions (Table V). Thus, the Tyr^1-Gly^2 amide bond is not important for an effective receptor interaction and can generally tolerate a certain degree of chemical modification without leading to a serious loss of biological activity. On the other hand, replacement of the Gly^2-Gly^3 bond by the methyleneoxy function resulted in a pseudopeptide analogue 17, which had low biological activity in the GPI and MVD bioassays and displayed low affinities for μ- and δ-binding sites in rat brain membrane assay.

Table VI. Relative Potencies of Several Pseudopeptide Analogues of [Leu^5]-, [Met^5]-, and [Pro^5]enkephalin Containing a Modified Gly^2-Gly^3 Bond

compound[a,b]	% rel potency
[Gly$^2\psi$(CH$_2$O)Gly^3,Leu^5]enkephalinamide	1.4
[Gly$^2\psi$(CH$_2$S)Gly^3,Leu^5]enkephalin	<1.0[c]
[Gly$^2\psi$(CSNH)Gly^3,Leu^5]enkephalin	400[d]
[Gly$^2\psi$(CH=CH)Gly^3,Pro^5]enkephalin	0.1[e]
[Gly$^2\psi$(CH$_2$CH$_2$)Gly^3,Met^5]enkephalinamide	<0.3[f]
[Gly$^2\psi$(CH$_2$NH)Gly^3,Met^5]enkephalinamide	0.3[f]
[NMeGly^3,Met^5]enkephalin	0.1[g]

[a] As determined in the electrically induced guinea pig ileum assay. [b] The notation (-)Xxx implies a modification of the peptide bond with the function that substitutes the amide bond between the parentheses. [c] Reference 8. [d] Reference 7. [e] Reference 10. [f] Reference 9. [g] Reference 1.

The importance of the Gly^2-Gly^3 amide bond for enkephalin activity has been demonstrated by several studies.[8-11,32] Hudson and co-workers[10] have proposed a conformational model for the binding of [Met]enkephalin to opiate receptors. In this model the peptide backbone is involved in receptor binding at only two points which are the N-terminal amino group of the Tyr^1 residue and the carbonyl group which is part of the Gly^2-Gly^3 amide bond. Fournie-Zaluski et al.[32] suggested a T-shaped structure similar to that of morphine for the low-energy conformers of the highest μ-selective peptides. Accordingly their model μ-selective peptides are obtained through replacement of Gly^2 by a hydrophobic amino acid strictly of the D configuration. However, replacement of Gly^2 by a hydrophilic residue will decrease μ specificity and contribute to a better δ-selective opioid peptide. The lack of biological activity for analogue 17 is in line with that observed for several other pseudopeptide analogues of enkephalin in which the Gly^2-Gly^3 peptide bond was replaced by a surrogate function (Table VI). The Gly^2-Gly^3 bond is, therefore, crucial for an effective interaction with the opiate receptor. Any structural modification at this site, except for the Ψ(CSNH) modification,[8] cannot be tolerated and invariably leads to an almost total loss of activity.

Experimental Section

Melting points were measured on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Optical rotations were measured on a Perkin-Elmer 141 automatic polarimeter. HPLC analysis was performed on a Merck-Hitachi liquid chromatograph, using a Whatman Partisil ODS RP-18 column (10 μM mean particle size, 0.4 × 25 cm), a Merck Hibar Lichrosorb RP-8 column (5 μM mean particle size, 0.5 × 25 cm), or a Merck Hibar Lichrosorb RP-18 column (5 μM mean particle size, 0.4 × 25 cm). Compounds were detected at 210 nm with the use of a Merck-Hitachi 655A-II variable-wavelength detector. TLC was performed on precoated silica gel plastic plates Polygram Sil NH-R/UV 254 from Macherey-Nagel Co. The solvent systems used were (A) CH_2Cl_2/MeOH/AcOH 17/2/1, (B) CH_2Cl_2/MeOH 2/1, (D) CH_2Cl_2/MeOH 19/1, (E) EtOAc/hexane 1/1. The plates were developed with the following reagent sprays: (1) ninhydrin, 4% in 1-BuOH, (2) fluorescamine (Fluram, Hoffman-La Roche & Co. AG), and/or (3) by chlorination with 1% *tert*-butyl hypochlorite followed by potassium iodide/*o*-tolidine,[34] or (4) Pauli

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Test.[35] Amino acid analyses were performed on LKB a 4400 amino acid analyzer, equipped with a Spectra-Physics 4100 printer/plotter computing integrator, using a four-component sodium buffer system. Hydrolysis of samples for amino acid analysis was carried out on 1-mg samples with constant-boiling HCl (0.5 mL), which was degassed, sealed at 0.1 mmHg, and heated for 24 h at 110 °C. The hydrolysate was dried under vacuum over KOH pellets and redissolved in sodium citrate buffer (0.5 mL), pH 3.2. Elementary microchemical analysis was carried out by Dr. S. Blum 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. Mass spectrometry was performed by Dr. K. Eckart at the laboratory of Prof. H. Schwartz, Department of Chemistry, The Technische Universitat, Berlin, Germany, using a fast atom bombardment (FAB) ion source. Preparative low-pressure liquid chromatography was performed on a Michel-Miller column system (ACE glass Inc.) using a Fluid Metering Inc. lab pump SY-2-CSC and silica gel 60, 0.04–0.063 mm (E. Merck), or Lichroprep RP-18, 40–60 μm (E. Merck).

Solvent and Reagent Purification. DMF was distilled under reduced pressure over NaH and redistilled from benzoic anhydride; CH_2Cl_2 and EtOAc were distilled over K_2CO_3; *N*-methylmorpholine (NMM) was distilled over KOH and redistilled in the presence of ninhydrin.

General Procedure for Peptide Couplings. To a stirred solution of Boc-amino acid (1.1–1.2 equiv) in DMF (0.4–0.7 M) was added 1 equiv of NMM. The solution was cooled to −25 °C under nitrogen, and isobutyl chloroformate (IBCF) (1.0–1.1 equiv) was added. After 2–5 min at −20 °C a precooled solution of the amine trifluoroacetate or hydrochloride component (1.0 equiv) in DMF (0.3–0.5 M) was added followed by NMM (1.0 equiv). After 10 min the pH of the reaction mixture was readjusted to pH 8.0–9.0 by adding NMM in 0.1-equiv portions.

Completion of the reaction was determined by negative fluorescamine test. After 1–2 h at −20 °C the reaction mixture was brought to 0 °C and a solution of 2 M $KHCO_3$ (3.0 equiv) was added and allowed to react for 0.5 h at 0 °C, which was then followed by solvent removal under reduced pressure. The residue obtained was redissolved in EtOAc which was washed consecutively with brine, 1 N $KHSO_4$, 5% $NaHCO_3$, and brine, dried over anhydrous $MgSO_4$, filtered, and evaporated to dryness under reduced pressure.

General Deprotection Procedure. Boc-protected peptides were treated with trifluoroacetic acid (20%–50% in methylene chloride) and in the presence of 1% (v/v) thioanisole, which prevented oxidation and alkylation of the sulfur atom of methionine and alkylation of the phenol moiety of tyrosine. The solution was kept for 30 min at room temperature protected with $CaCl_2$. Solvent was removed under reduced pressure and the residue was dried under vacuum over KOH. The residue obtained was then triturated with dry ether and collected by filtration. The solid was dried under vacuum over P_2O_5.

Materials. [pGlu^6]P_{6-11}, [pGlu^6,NMePhe^8]P_{6-11}, and [pGlu^6,Pro^9]P_{6-11} were synthesized as described elsewhere.[2,3] [D-Ala^2,Met^5]enkephalinamide was purchased from the Peptide Institute (Osaka, Japan). [D-Ala^2,NMePhe^4,Gly^5-ol]enkephalin (DAGO), [D-Ala^2,D-Leu^5]enkephalin (DADLE), and [D-Ser^2,Leu^5]enkephalyl-Thr^6 (DSLET) were purchased from Bachem A.G. Naloxone was purchased from Du Pont.

Boc-PheΨ(CH$_2$O)Gly-OH (2). Ester 1[18] (0.8 g, 2.37 mmol) was dissolved in dioxane (23.7 mL). To the solution at 0 °C was added 0.2 N NaOH (23.7 mL). The reaction mixture was stirred at room temperature for 1 h, followed by evaporation of the solvent under reduced pressure. The residue was dissolved in 100 mL of water and extracted with ether. The aqueous phase was acidified with 2 N $KHSO_4$ to pH 2–3 and extracted with EtOAc (3 × 100 mL). The combined EtOAc fractions were washed once with brine and were dried over $MgSO_4$. Filtration and evaporation of the solvent under reduced pressure gave the product as a clear oil: yield 0.7 g (96%) of **2**. The product was shown to be homogenous by TLC [R_f (C) 0.67, (E) 0.20].

Boc-PheΨ(CH$_2$O)Gly-Leu-Met-NH$_2$ (3). Compound **2** (0.565 g, 1.83 mmol) in dry DMF (3.6 mL) was reacted with IBCF (0.22 mL, 1.75 mmol) in the presence of NMM (0.193 mL, 1.75 mmol) at −25 °C. TFA·H-Leu-Met-NH_2 (0.60 g, 1.59 mmol) in DMF (3.2 mL) was added to the reaction mixture followed by NMM (0.148 mL, 1.59 mmol). The reaction mixture was worked up according to the standard procedure described above. The crude product was recrystallized from EtOAc/hexane to give 0.60 g (75%) of pseudopeptide **3**: mp 88–91 °C; $[\alpha]^{25}_D$ −28 °C (c = 1.0, MeOH); TLC R_f (C) 0.64, (D) 0.35; HPLC k' (MeOH/H_2O) 2.00 (75/25), 5.50 (65/35). Anal. ($C_{27}H_{44}N_4O_6S$) C, H, N.

Boc-Phe-PheΨ(CH$_2$O)Gly-Leu-Met-NH$_2$ (5). The N-protected pseudotetrapeptide **3** (0.56 g, 1.01 mmol) was deprotected according to the standard procedure described above. The precipitate obtained from dry ether was dried under vacuum to give 0.56 g (97%) of **4**. The product was shown to consist of a single spot on this TLC: R_f (A) 0.58, (D) 0.29.

Boc-Phe-OH (0.296 g, 1.12 mmol) in dry DMF (2.2 mL) was reacted with IBCF (0.11 mL, 1.07 mmol) in the presence of NMM (0.14 mL, 1.07 mmol) at −25 °C. The amine component TFA·H-PheΨ(CH$_2$O)Gly-Leu-Met-NH_2 (**4**) (0.55 g, 0.97 mmol) in DMF (1.9 mL) was added to the reaction mixture followed by NMM (0.11 mL, 0.97 mmol). The reaction mixture was worked up according to the standard procedure described above. The crude product was recrystallized from EtOAc/hexane to give 0.515 g (70%) of pure pseudopentapeptide **5**: mp 176–178 °C; $[\alpha]^{25}_D$ −21.3° (c = 1.0, MeOH); TLC R_f (C) 0.71, (D) 0.62; HPLC k' (MeOH/H_2O) 3.37 (75/25), 4.18 (70/30). Anal. ($C_{36}H_{53}N_5O_7S$) C, H, N.

pGlu-Phe-PheΨ(CH$_2$O)Gly-Leu-Met-NH$_2$ (7). N-Protected pseudopentapeptide **5** (0.47 g, 0.67 mmol) was deprotected according to the standard procedure described above. The solid obtained from dry ether was dried in vacuo: yield 0.51 g (98%) of **6**. The product was shown to be pure by TLC [R_f (A) 0.38, (C) 0.34, (D) 0.11]. pGlu-OH (0.185 g, 1.43 mmol) in dry DMF (2.8 mL) was reacted with IBCF (0.169 mL, 1.3 mmol) in the presence of NMM (0.143 mL, 1.3 mmol) at −25 °C. The amine component TFA·H-Phe-PheΨ(CH$_2$O)-Gly-Leu-Met-NH_2 (**6**) (0.51 g, 0.714 mmol) in DMF (1.4 mL) was added to the reaction mixture followed by NMM (0.79 mL, 0.714 mmol). The reaction mixture was worked up according to the standard procedure described above. The crude product was recrystallized from EtOAc/hexane, to give 0.363 g (72%) of pseudohexapeptide **7**: mp 170–174 °C; $[\alpha]^{25}_D$ −28.5° (c = 1.0, MeOH); TLC R_f (C) 0.58, (D) 0.18; HPLC k' (MeOH/H_2O) 0.86 (75/25), 3.45 (65/35); FAB-MS m/e 711 (M + H)$^+$; amino acid analysis, Glu 1.00, Phe 0.99, Leu 1.05, Met 0.94. Anal. ($C_{36}H_{50}N_6O_7S$) C, H, N.

Z-Tyr(Bzl)Ψ(CH$_2$O)Gly-OH (9). Ester 8[18] (0.3 g, 0.59 mmol) was stirred at room temperature in 25% TFA in CH_2Cl_2 (10 mL). The reaction was monitored by TLC. After 1 h no starting material was detected. The solvents were evaporated in vacuo, and the oily residue was kept under high vacuum over KOH. Recrystallization from EtOAc/hexane gave 0.2 g (76%) of pseudodipeptide **9**. The product was shown to be pure by TLC [R_f (A) 0.68, (D) 0.28, (E) 0.15]: mp 125 °C; $[\alpha]^{25}_D$ −24.6° (c = 0.5, MeOH).

Z-Tyr(Bzl)Ψ(CH$_2$O)Gly-Gly-Phe-Leu-NH$_2$ (10). Compound **9** (0.188 g, 0.42 mmol) in dry DMF (0.8 mL) was reacted with IBCF (0.052 mL, 0.4 mmol) and in the prescence of NMM (0.044 mL, 0.4 mmol) at −25 °C. The amine component TFA·H-Gly-Phe-Leu-NH_2 (0.179 g, 0.40 mmol) in DMF (0.8 mL) was added to the reaction mixture followed by NMM (0.44 mL, 0.4 mmol). The reaction mixture was worked up according to the standard procedure described above. The crude product was dissolved in hot EtOAc, filtered to remove insoluble impurities, and was crystallized by addition of petroleum ether (bp 35–60 °C) to give a white solid: yield 0.23 g (75%) of the protected pseudopentapeptide **10**; mp 184–186 °C; $[\alpha]^{25}_D$ −30.5° (c = 1.0, MeOH); TLC R_f (C) 0.84, (D) 0.22; HPLC k' (MeOH/H_2O) 1.37 (70/30), 2.00 (65/35), 3.02 (60/40). Anal. ($C_{43}H_{51}N_5O_8$) C, H, N.

H-TyrΨ(CH$_2$O)Gly-Gly-Phe-Leu-NH$_2$ (11). N,O-Diprotected pseudopentapeptide **10** (0.063 g, 0.08 mmol) was dissolved in a mixture of MeOH/AcOH (3/1) (5 mL). To this mixture, flushed with N_2, were added an excess of ammonium formate (20 mg, 0.3 mmol) and 10% Pd/C (12 mg). The reaction mixture

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(35) Von Arx, E.; Meher, R. J. *J. Chromatogr.* **1963**, *12*, 329.

was stirred for 15 h at room temperature. Completion of the reaction was followed by HPLC analysis of the reaction mixture. After removal of Pd/C by filtration through a Celite pad, the solvent was removed under reduced pressure followed by lyophilization of the residue twice, from a mixture of H_2O/AcOH to remove any traces of ammonium formate. Pseudopentapeptide 11 (acetate form) was obtained as a white powder: yield 47 mg (98%); mp 75–79 °C; $[\alpha]^{25}_D$ −12.5° (c = 1.0, MeOH); TLC R_f (C) 0.19, (A) 0.51, (B) 0.66; HPLC k' (MeOH/H_2O) 0.62 (65/35), 0.87 (60/40), 1.75 (50/50); FAB-MS m/e 542 $[M + H]^+$; amino acid analysis, Gly 1.00, Phe 1.00, Leu 0.98. Anal. ($C_{28}H_{39}N_5O_6 \cdot CH_3COOH \cdot 2.0H_2O$) C, H, N.

Pht-GlyΨ(CH_2O)Gly-OH (13). Ester 12[22] (3.8 g, 12.46 mmol) was dissolved in CH_2Cl_2 (30 mL) followed by addition of TFA (8 mL) at 0 °C. The reaction mixture was stirred for 1.5 h at room temperature under $CaCl_2$ protection. The solvents were evaporated under reduced pressure and the oily residue was kept overnight under high vacuum over KOH. The solid was recrystallized from 2-propanol/diisopropyl ether, upon standing in the cold room. The product was obtained as white-clear crystals: yield 2.4 g (78%) of deprotected pseudodipeptide 13; mp 129–131 °C, R_f (A) 0.75, (C) 0.23, (E) 0.15; EI-MS $(m/e)^+$ 249 [M], 250 [M + H]. Anal. ($C_{12}H_{11}NO_5$) C, H, N.

Pht-GlyΨ(CH_2O)Gly-Phe-Leu-NH_2 (14). Compound 13 (1.2 g, 4.82 mmol) in dry DMF (10 mL) was reacted with IBCF (0.6 mL, 4.6 mmol) and in the presence of NMM (0.53 mL, 4.6 mmol), at −25 °C. The amine component HCl·H-Phe-Leu-NH_2 (1.37 g, 4.42 mmol) in DMF (9 mL) was added to the reaction mixture followed by NMM (0.48 mL, 4.42 mmol). The reaction mixture was worked up according to the procedure described above. The product was recrystallized from a mixture of EtOAc/diethyl ether and petroleum ether (bp 35–60 °C) to give 2.2 g (98%) of protected pseudotetrapeptide 14: mp 218–221 °C dec; $[\alpha]^{25}_D$ −11.2° (c = 1.0, DMF); TLC R_f (C) 0.68, (D) 0.32; HPLC k' (MeOH/H_2O), 1.62 (70/30), 5.4 (60/40); FAB-MS $(m/e)^+$ 509 [M + H]. 492 [M + H − NH_3]. Anal. ($C_{27}H_{32}N_4O_6$) C, H, N.

Boc-Tyr-GlyΨ(CH_2O)Gly-Phe-Leu-NH_2 (16). N^{α}-Protected pseudotetrapeptide 14 (2.0 g, 3.94 mmol) was dissolved in MeOH (40 mL). To the solution were added glacial AcOH (0.45 mL, 7.87 mmol) and $N_2H_4 \cdot H_2O$ (0.39 g, 7.87 mmol). The reaction mixture was refluxed under N_2 and the progression of the reaction was followed by TLC and HPLC. After 6 h no starting material was detected. The reaction mixture was cooled to 0 °C and the phthalohydrazide precipitate was filtered off. The filtrate was evaporated under reduced pressure and the residue was redissolved in a mixture of cold MeOH/AcOH. The residual insoluble material was filtered and the filtrate evaporated under reduced pressure. The residue was lyophilized twice from a mixture of AcOH/H_2O to give 1.4 g (82%) of the acetate form of 15 as a pale-yellow hygroscopic crystalline material: mp 49–53 °C; TLC R_f (C) 0.35; HPLC k' (MeOH/H_2O) 0.73 (70/30). Boc-Tyr-OH (0.38 g, 1.35 mmol) in dry DMF (2.7 mL) was reacted with IBCF (0.170 mL, 1.3 mmol) and in the presence of NMM (0.143 mL, 1.3 mmol), at −25 °C. The amine component AcOH·H-GlyΨ(CH_2O)Gly-Phe-Leu-NH_2 (0.54 g, 1.23 mmol) in DMF (2.46 mL) was added to the reaction mixture followed by NMM (0.136 mL, 1.23 mmol). The reaction mixture was worked up according to the standard procedure described above. The crude product was further purified by reverse-phase (RP-18) low-pressure LC by using a gradient of MeOH in H_2O (50%–75% MeOH/H_2O). The main fraction which eluted along with 55%–60% MeOH/H_2O and was lyophilized to give 0.325 g (41%) of protected pseudopentapeptide 16: mp 105–109 °C; $[\alpha]^{25}_D$ −9.4° (c = 1.0, MeOH); TLC R_f (C) 0.48, (D) 0.23; HPLC k' (MeOH/H_2O) 1.67 (75/25), 3.25 (65/35), 4.5 (60/40); amino acid analysis Tyr 1.00, Phe 1.05, Leu 1.09. Anal. ($C_{33}H_{47}N_5O_8 \cdot H_2O$) C, H, N.

TFA·H-Tyr-GlyΨ(CH_2O)Gly-Phe-Leu-NH_2 (17). Boc-N^{α}-protected pseudopentapeptide 16 (0.27 g, 0.421 mmol) was deprotected according to the method described above. The residue was recrystallized twice from MeOH/ether to give 103 mg (46%) of pseudopentapeptide 17: mp 69–73 °C; $[\alpha]_D$ −15.6° (c = 1.0, MeOH); TLC R_f (A) 0.13, (B) 0.82; HPLC k' (MeOH/H_2O) 0.48 (70/30), 0.59 (65/35), 0.77 (55/45); FABS-MS $(m/e)^+$ 542 [M + H]; amino acid analysis, Tyr 0.98, Phe 1.00, Leu 1.00. Anal. ($C_{28}H_{39}N_5O_5 \cdot CF_3COOH \cdot H_2O$) C, H, N.

Biological Assays. The isolated guinea pig ileum assay was carried out as previously described.[23,27] Briefly, the isolated GPI preparation was suspended in a 10-mL organ bath containing Tyrode solution (composition in mM: 118 NaCl, 4.7 KCl, 1.8 $CaCl_2$, 0.5 $MgCl_2$, 1.0 NaH_2PO_4, 25 $NaHCO_3$, and 10 glucose), gassed with a 95% O_2/5% CO_2 mixture and maintained at 34 °C. Contractions were recorded isotonically. Peptides were applied at 2–3-min intervals with less than 30 s contact time. At the beginning of a desensitization experiment, the GPI was first incubated with a high dose (10^{-7} M) agonist until the response had faded to the baseline (2–4 min). The tissue was then washed and immediately reincubated with the agonist (10^{-7} M) for 2 min. The contraction caused by a test peptide was then recorded. All test peptides produced similar maximal contractions in a given test preparation. Relative potencies were calculated from EC_{50} values (concentration of agonist producing 50% of the maximal contraction).

Potentiation of electrically stimulated rat vas deferens (RVD) contraction was determined as described by Lee et al.[26]

The assay based on inhibition of electrically induced contractions of the GPI by opiate agonists was carried out as described by Kosterlitz et al.[36]

The mouse vas deferens (MVD) assay was essentially performed as described elsewhere.[37] Adult, male mice (C_{57}/BL, 28–30 g) were killed by cervical dislocation and the vas deferentia were dissected out. After removal of extraneous fat and connective tissue each vasa was stripped of its associated blood vessel and the semen was gently expressed from the lumen. A pair of vasa was ligated together and then mounted under 0.8-g tension in a 10-mL organ bath containing a Mg^{2+}-free Krebs solutions of the following composition (mM): 118 NaCl, 4.75 KCl, 2.52 $CaCl_2$, 1.17 KH_2PO_4, 25 $NaHCO_3$, and 11 glucose, gassed with a 95% O_2/5% CO_2 mixture and warmed to 37 °C. A Grass 88 apparatus stimulator delivered repetitive field stimulation through platinum wire electrodes placed in parallel (5 mm apart) from the vasa. The stimuli consisted of rectangular pulses (54 V, 0.12 Hz, 1.00-ms duration). Contractions of the muscle were recorded via a Hugo Sachs Model 305 HF-Modem force transducer connected to a four-channel Hugo-Sachs transducer. Peptides were applied at 10–15-min intervals with less than 60-s contact time. Determination of the reduction in the twitch height at various doses (six to eight for each peptide) permitted the construction of log dose–response curves. A log dose–response curve was determined with [D-Ala^2,Leu^5]enkephalinamide as standard for each GPI or MVD preparation. The rat brain membrane (RBM) binding assay was performed in triplicate essentially as described in ref 38. For data analysis the inhibitory constant K_i of the various nonradioactive compounds was calculated from the Cheng and Prusoff equation,[39] $K_i = IC_{50}/(1 + L/K_d)$ (where IC_{50} is the concentration of the inhibitor that decreased the binding of the radioligand by 50%, L is the concentration of the labeled ligand, and K_d is the equilibrium dissociation constant). The dissociation constant K_d for the ligands was determined from least-squares linear-regression analysis of Scatchard plots.

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(40) Abbreviations according to IUPAC–IUB Joint Commission on Biochemical Nomenclature (*Eur. J. Biochem.* **1984**, *138*, 9). The following special abbreviations are used for the methyleneoxy pseudopeptides and fragments: Two standard three-letter notations for amino acid residues separated by Ψ(CH_2O) represent a pseudodipeptide modified by a methyleneoxy peptide bond surrogate. Other abbreviations are Boc, tert-butyloxycarbonyl; Z, benzyloxycarbonyl; Pht, phthalimide; Bzl, benzyl; OBu^t, t-butyl ester; TFA, trifluoroacetic acid; CH_2Cl_2, dichloromethane; TA, thioanisole; NMM, *N*-methylmorpholine; DMF, *N*,*N*-dimethylformamide; MeOH, methanol; and AcOH, glacial acetic acid.

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Registry No. 1, 98900-25-5; 2, 60398-45-0; 3, 133851-82-8; 4·TFA, 133851-84-0; 5, 133851-85-1; 6·TFA, 133851-87-3; 7, 98900-29-9; 8, 109953-94-8; 9, 133851-88-4; 10, 133851-89-5; 11·AcOH, 133851-91-9; 11 (free base), 133851-90-8; 12, 98900-24-4; 13, 69676-65-9; 14, 133851-92-0; 15·AcOH, 133851-94-2; 16, 133851-95-3; 17·TFA, 133907-21-8; 17 (free base), 98900-28-8; BOC-Phe-OH, 13734-34-4; H-pGlu-OH, 98-79-3; H-Gly-Phe-Leu-NH_2·TFA, 133851-96-4; H-Phe-Leu-NH_2·HCl, 81638-86-0; BOC-Tyr-OH, 3978-80-1; H-Leu-Met-NH_2·TFA, 84552-48-7.

Electrophilic γ-Lactone κ-Opioid Receptor Probes. Analogues of 2′-Hydroxy-2-tetrahydrofurfuryl-5,9-dimethyl-6,7-benzomorphan Diastereomers

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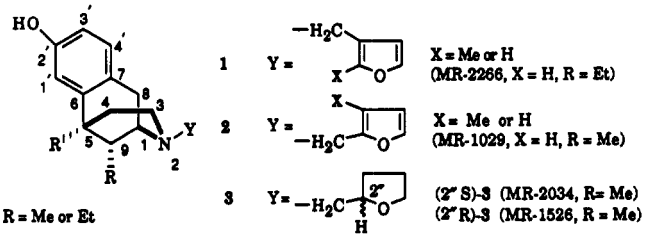
Benzomorphans with an electrophilic group in the nitrogen substituent were prepared as potentially irreversible ligands for the κ-opioid receptor. These were synthesized from products of the reaction of normetazocine with the enantiomers of 5-(iodomethyl)-γ-butyrolactone (11). α-Methylene γ-lactones **5** and **7** and endocyclic α,β-unsaturated γ-lactones **8** and **9** were prepared from the corresponding saturated γ-lactones **13** and **23** possessing the "active" (1*R*,5*R*,9*R*)-benzomorphan stereochemistry. Only γ-lactones **8**, **9**, **13**, and **23**, lacking the exocyclic methylene group, retain significant affinities for opioid receptor binding sites when compared with the reference compounds (2″*S*)-**3** and (2″*R*)-**3**. As observed with these references compounds, greater binding affinity is also seen with γ-lactone diastereomers having the 2″*S* stereochemistry in the nitrogen substituent. Although the γ-lactones do not bind irreversibly in opioid receptor preparations, they do show κ-receptor selectivities comparable to those observed for the reference compounds.

A series of *N*-(furanylmethyl)- and *N*-tetrahydrofurfurylbenzomorphans (**1**–**3**), reported by Merz and coworkers,[1-5] have very interesting pharmacological profiles. Among these are the *N*-furanylmethyl series of compounds (**1** and **2**) and related compounds which, depending on the location of the attachment of the substituted or unsubstituted furan ring, show mixed agonist antagonist effects or even agonist effects, but have no ability to substitute for morphine in dependent monkeys. The corresponding *N*-2-furanylethyl homologue is a potent analgesic (25 × morphine in the writhing assay).[1,6] In the more recently reported *N*-tetrahydrofurfuryl series (**3**), introduction of a new chiral center in the N-substitutent greatly influences activity. The new 2″*S* chiral center confers primarily potent analgesic properties (up to 50 × morphine on a molar basis) and no ability to substitute for morphine in dependent monkeys to (2″*S*)-**3** (R = Me), while its diastereomer with the 2″*R* chiral center had primarily antagonist properties.[4,5] Compound (2″*R*)-**3** (R = Me) showed weak analgesic properties in the writhing assay and was not analgesic in the tail-clip and hot-plate assays. The effects of these agents have been assigned primarily to interaction at the κ-receptor subclass of opioid receptors where **1** (X = H, R = Et, MR-2266) is believed to be a selective antagonist.[7-9] Although the *N*-tetrahydrofurfuryl compounds displace κ-receptor ligands, they also displace μ-receptor ligands.



Based on this information, we prepared the diastereomeric N-substituted α-methylene γ-lactone derivatives that have an electrophilic group incorporated into the tetrahydrofurfuryl substituent in order to obtain possible affinity ligands for κ-receptors. We thought that replacement of the (2″*S*)-tetrahydrofurfuryl substituent on the basic benzomorphan nitrogen with the analogous (2″*S*)-α-methylene γ-lactone substituent (as in **5**) would provide minimal structural distortion, thereby maintaining a high degree of affinity for opioid binding sites, while simultaneously incorporating a potentially useful electrophile[10-13]

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