hydroxy-5-methoxybenzoyl)piperidine oxime acetate, 95742-17-9; 4-(2-hydroxy-4-methoxybenzoyl)piperidine hydrochloride, 84162-88-9; 4-(2-hydroxy-4-methoxybenzoyl)piperidine, 64671-19-8; phenylmethyl 4-(2-hydroxy-4-methoxybenzoyl)-1-piperidinecarboxylate, 84162-91-4; phenyl 4-(1,2-benzisoxazol-3-yl)-1-piperidinecarboxylate, 84163-21-3; 4-chloro-1,1-bis(4-fluorophenyl)butane, 3312-04-7; 2-methyl-3-[3-(phenylsulfonyl)propyl]indole, 92933-14-7; 3-(3-chloropropyl)-6-fluoro-1,2-benzisoxazole, 84243-02-7; 3-(1-cyano-4-piperidinyl)-4-fluoro-1,2-benzisoxazole, 95742-18-0; 4-fluoro-3-(4-piperidinyl)-1,2-benzisoxazole hydrochloride, 95742-19-1; 4-fluoro-3-(4-piperidinyl)-1,2-benzisoxazole, 95742-20-4; 1-(3-chloropropyl)-1,3-dihydro-(2*H*)-benzimidazol-2-one, 62780-89-6; 2-fluorobenzonitrile, 394-47-8; 2,6-difluorobenzonitrile, 1897-52-5; 1,3-difluorobenzene, 372-18-9; 1,3-dichlorobenzene, 541-73-1; 4-fluoroanisole, 459-60-9; 1,4-dimethoxybenzene, 150-78-7; 1,3-dimethoxybenzene, 151-10-0; acetic formic anhydride, 2258-42-6; isonipecotic acid, 498-94-2; hydroxylamine hydrochloride, 5470-11-1; benzyl chloroformate, 501-53-1; phenyl chloroformate, 1885-14-9.

Synthesis and Pharmacological Activity of Partially Modified Retro-Inverso Dermorphin Tetrapeptides†

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We studied the effect of partial retro-inverso modification of selected peptide bonds of N-terminal tetrapeptide analogues of dermorphin (H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2). Among the 14 compounds synthesized and tested for opioid activity, some tetrapeptides have the C-terminus carrying different amide moieties; retromodifications concern the Phe–Gly bond (Ia–f) and/or the C-terminal carboxamide function (IIIa–d, IIa–d). All pseudotetrapeptide derivatives showed opioid activity in vitro and in vivo. The most potent compounds (II) have a biological potency comparable with that of the original tetrapeptides in the guinea pig ileum preparation and in the mouse tail-flick test after intracerebral or subcutaneous administration.

Dermorphins are opioid heptapeptides isolated from the skin of some South American frogs.[1] Their sequences were established to be the following:[2,3]

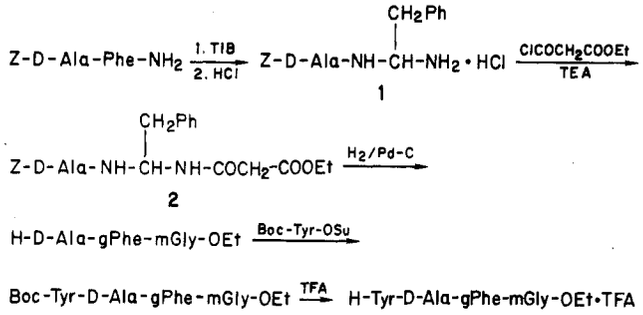
H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2 (D)
H——————————Hyp——NH_2 (HD)

Synthetic dermorphins, identical with the natural peptides have been prepared by various methods, using solution[4] or solid-phase[5] techniques. Opioid peptides D and HD possess peripheral and central activity: morphine-like effects in rats,[6] mice[4b,5,7] cell-line preparations,[8] and man[9] have been reported. Our earlier investigation on D analogues concerned the synthesis and study of structure–activity relationships[10] of 60 "small dermorphins" and the synthesis of tetrapeptides (H-Tyr-D-Ala-Phe-Gly-NH-Y), which are very potent analgesics after intracerebroventricular (icv) injection but not following subcutaneous (sc) administration.[11]

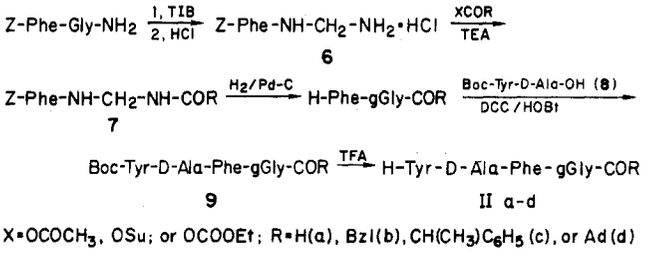
This drawback may result from unfavorable pharmacokinetic or transport parameters and/or low stability in plasma; cleavage of Phe–Gly and Gly–Tyr bonds was postulated to be the main biodegradation pathway of D.[12]

We undertook the synthesis of new D tetrapeptide analogues in which one or more amide bonds are reversed, in the expectation that these partially modified retro-inverso (PMRI)[13] isomers would exhibit enhanced stability toward enzymic degradation. Furthermore, the new

Scheme I. Synthesis of PMRI Analogues Ia–l



Scheme II. Synthesis of PMRI Analogues IIa–d



† Abbreviations according to the IUPAC–IUB Commission, *Biochemistry*, **1972**, *11*, 1726–1732, and Specialist Periodical Reports, "Amino Acids, Peptides and Proteins", The Chemical Society, London, 1980, Vol. 11, 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 prefix g represents the *gem*-diaminoalkyl 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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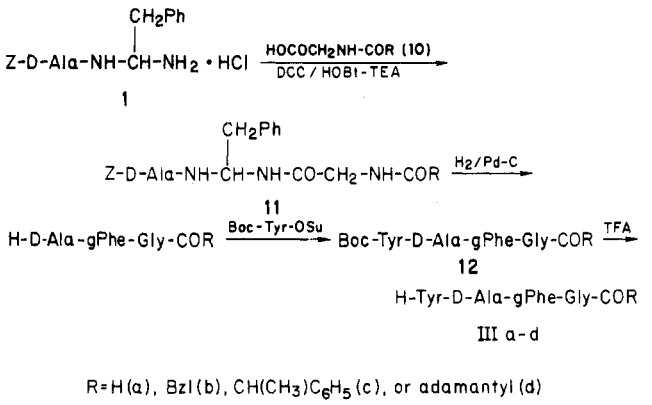
Scheme III. Synthesis of PMRI Analogues IIIa–d

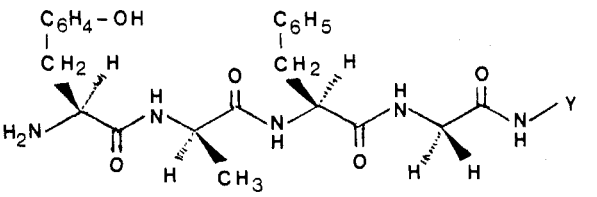


pseudotetrapeptides have the carboxy termini carrying different amide moieties.

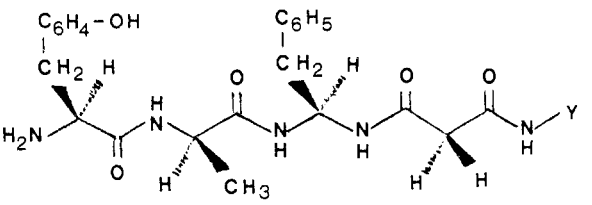
A number of partially retro-inverso peptide hormones has been recently investigated.[14] Figure 1 shows the analogues and their structural relationships to the parent dermorphin tetrapeptides (DTs): the Phe–Gly bond, considered the most susceptible to proteolityc cleavage, is reversed in Ia–d; the terminal carboxamide function is reversed in IIa–d; and both amide bonds are reversed in IIIa–d. A retro-inverso isomer carrying a terminal free carboxyl group (Ie) and its ethyl ester (If) were also obtained (see Scheme I).

Results

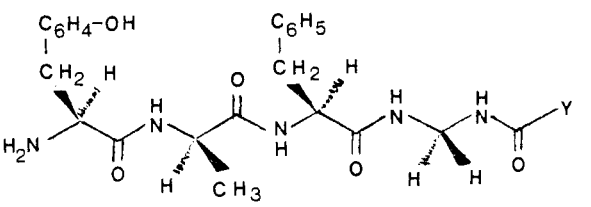
Chemistry. Schemes I–III outline the preparation of [gPhe,mGly]DTs (Ia–f), [gGly]DTs (IIa–d), and [gPhe,-Gly]DTs (IIIa–d). The *gem*-diaminoalkyl moieties of



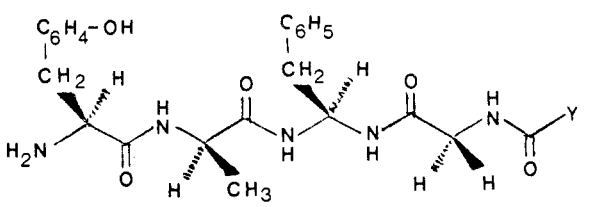
DTs (a-d)



[gPhe,mGly]DTs I (a-d)



[gGly]DTs II (a-d)



[gPhe,$\overleftarrow{\text{Gly}}$]DTs III (a-d)

Y = H (a); Bzl (b); CH(CH3)C6H5 (c); Ad (d)

Figure 1. Structures of parent dermorphin tetrapeptides (DTs) and PMRI analogues [gPhe,mGly]DTs (Ia–d), [gGly]DTs (IIa–d), and [gPhe,$\overleftarrow{\text{Gly}}$]DTs (IIIa–d).

compounds **1** and **6** were obtained by treating the appropriate benzyloxycarbonyl dipeptide amide with bis(trifluoroacetoxy)iodobenzene (TIB)[15] under conditions known to proceed without racemization.[15b] Coupling with the malonyl ester chloride (Scheme I), an acyl chloride (Scheme II), or acylglycine (Scheme III) resulted in the protected PMRI pseudopeptide units **2**, **7**, or **11**. Depro-

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Table I. Physicochemical Properties of PMRI Analogues of DTs

no.	R	mp, °C	$[\alpha]^{24}_D$	R_f[a]	formula	anal.
			H-Tyr-D-Ala-gPhe-mGly-R			
Ia	NH_2	213–215	+27.8[b]	0.52	$C_{23}H_{29}N_5O_5 \cdot C_2H_4O_2$	C, H, N
Ib	NH-$CH_2C_6H_5$	130–132	+19.6[b]	0.55	$C_{30}H_{35}N_5O_5 \cdot C_2H_4O_2$	C, H, N
Ic	D-NHCH(CH_3)C_6H_5	176–178	+74.1[c]	0.59	$C_{31}H_{37}N_5O_5 \cdot C_2HF_3O_2$	N
Id	NH-Ad	122–124	+27.5[b]	0.75	$C_{33}H_{43}N_5O_5 \cdot C_2HF_3O_2$	C, H, N
Ie	OH	164–166	+77.7[b]	0.46	$C_{23}H_{28}N_4O_6$	C, H, N
If	OEt	148–150	+52.6[c]	0.53	$C_{25}H_{32}N_4O_6 \cdot C_2H_4O_2$	N
			H-Tyr-D-Ala-Phe-gGly-R			
IIa	COH	131–133	+40.6[c]	0.53	$C_{23}H_{29}N_5O_5 \cdot C_2H_4O_2$	C, H, N
IIb	$COCH_2C_6H_5$	129–131	+31.9[c]	0.56	$C_{30}H_{35}N_5O_5 \cdot C_2H_4O_2$	N
IIc	D-COCH(CH_3)C_6H_5	138–140	+9.3[d]	0.60	$C_{31}H_{37}N_5O_5 \cdot C_2H_4O_2$	C, H, N
IId	CO-Ad	135–138	+16.5[d]	0.74	$C_{33}H_{43}N_5O_5 \cdot C_2HF_3O_2$	N
			H-Tyr-D-Ala-gPhe-Gly-R			
IIIa	COH	130–132	+63.9[d]	0.53	$C_{23}H_{29}N_5O_5 \cdot C_2HF_3O_2$	N
IIIb	$COCH_2C_6H_5$	152–154	+26.9[b]	0.57	$C_{30}H_{35}N_5O_5 \cdot C_2H_4O_2$	C, H, N
IIIc	D-COCH(CH_3)C_6H_5	135–137	+37.8[d]	0.59	$C_{31}H_{37}N_5O_5 \cdot C_2H_4O_2$	N
IIId	CO-Ad	158–160	+49.6[d]	0.73	$C_{33}H_{43}N_5O_5 \cdot C_2HF_3O_2$	C, H, N

[a] Thin-layer chromatography on silica gel: solvent system (B). [b] *c* 0.5 in acetic acid. [c] *c* 1.0 in acetic acid. [d] *c* 1.0 in methanol.

tection, followed by coupling with the appropriate N-terminal fragment yielded the fully protected, partially modified retro-inverso analogues **3**, **9**, and **12**. Hydrolysis of ester **3** and further derivatization via DCC/HOBt gave PMRI amides **5**. Treatment of compounds **3**, **5**, **9**, and **12** with trifluoroacetic acid gave the desired analogues (Figure 1 and Schemes I–III).

Final purification was accomplished by partition chromatography with solvent system A on Sephadex G-25. The purity of I–III was ascessed by TLC, HPLC, amino acid analysis, and elemental microchemical analysis (see Table I and the Experimental Section).

Biological Activity. The PMRI DT analogues were tested in vitro by the electrically stimulated guinea pig ileum (GPI) preparation[16] and in vivo by the mouse tail-flick[17] assay after intracerebroventricular (icv) and subcutaneous (sc) administration. The pharmacological results of 14 new DT analogues were reported in Table II, in comparison with morphine, dermorphin, and the parent peptides (DTs).[10a] In the GPI test the potency of compounds Ia–f is one-third to one-fifth that of the closest related analogues with a nonmodified backbone DTa–f. Pseudopeptides IIa–d are more potent than the corresponding parent tetrapeptides DTa–d: IId, the most active compound of the series, is 200 and 5 times more potent than morphine or dermorphin, respectively. On the other hand, the PMRI analogues IIIa–d, containing both retro-inversions, showed only 10% potency with respect to the original tetrapeptides.

The activity of all compounds was completely reversed by the spcific opiate-receptor antagonist, naloxone hydrochloride, at 2.5 nM concentration. In the tail-flick test, after icv administration, the influence of retro-inverso modification on DTs generally matches the results of in vitro assay: whereas retro-inverso isomers Ia–d and IIIa–d displayed comparatively low antinociceptive effects, analogues IIa–d containing a retro-inversion of the terminal carboxamide function showed potent analgesic activity (even if slightly lower than original DTa–d).

In contrast with icv results, all pseudopeptides displayed rather low activity after sc administration: prototype IId was weaker than its parent DTs, as well as weaker than morphine or dermorphin. The analgesic activity of all investigated peptides was abolished by sc administration of naloxone hydrochloride (0.5–1 mg/kg, 5 min prior to the testing procedure).

Discussion

Generally, the retro-inverso modification of peptide bond(s) results in different folded conformations for the parent peptide and its PMRI analogues.[14b,18] Therefore, the data presented here indicate that the majority of pseudotetrapeptides retains conformational requirements for opioid activity; the reversal of the original Phe–Gly and/or terminal carboxamide bonds is, in fact, tolerated in the GPI assay. Furthermore, all the PMRI isomers tested show dose-related naloxone-reversible analgesic efficacy in vivo. Thus, it is apparent that the structures of parent peptides and their pseudopeptide analogues I–III share topochemical and, at least in part, spatial resemblance. The closest structural complementarity of both the side chains and end groups appears to pertain to the most active compounds IIa–d, modified at the terminal carboxamide bond. In the less active analogues Ia–f and/or IIIa–d, on the contrary, the reversal of Phe–Gly peptide bond may perturb important interactions (i.e., hydrogen bonds) with the receptors or within the molecule. In this respect, it is interesting to point out that in DTs the substitution of CH_2 for NH at the Phe–Gly bond causes substantial reduction of biological activity.[19]

The analgesia subsequent to systemic administration of PMRI analogues deserves separate consideration. Following sc injection, analgesia from IIb–d was similar in duration ($t_{1/2}$ in the range of 45–65 min) and potency to that observed for the parent DTs but relatively weaker than expected. None of these PMRI analogues, after sc administration, is as active as the potency by the icv route would suggest. A difference in the metabolic stability cannot fully explain the difference: the presence of D-Ala[2] and the modification of labile linkages in DTs might, in fact, protect them from enzymatic cleavage.[20] Thus, low analgesic potency after sc injection can be due to inadeguate CNS permeation, but other factors (diffusion from subcutaneous sites, entry into blood, and binding to plasma proteins) may be more important than metabolic stability.

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Table II. Biological Activities of PMRI Analogues of Dermorphin Tetrapeptides

no.	compd	GPI:[a] IC_{50}, nM	analgesia (icv):[b] ED_{50}, pM/mouse	$t_{1/2}$, min	analgesia (sc):[b] ED_{50}, μM/kg	$t_{1/2}$, min	rel potencies[c] GPI	analgesia icv	analgesia sc
Ia	H-Tyr-D-Ala-gPhe-mGly-NH_2	242.5 ± 16.5	974.5 (730.8–1450)	35			0.24	3.11	
Ib	H——NH-$CH_2C_6H_5$	18.2 ± 1.4	555.7 (391.8–744.5)	35			3.21	5.45	
Ic	H——D-NHCH(CH_3)C_6H_5	2.05 ± 0.13	88.6 (65.7–117.3)	65	44.2 (29.1–70.3)	60	28.61	34.19	0.24
Id	H——NH-Ad	4.45 ± 0.44	88.4 (60.3–115.6)	65	55.5 (26.3–97.3)	50	13.18	34.26	0.19
Ie	H——OH	330.0 ± 25.4	1605 (1222–2452)	30			0.17	1.88	
If	H——OEt	320.0 ± 21.2	1629 (881.1–1834)	30			0.18	2.38	
IIa	H-Tyr-D-Ala-Phe-gGly—COH	39.2 ± 2.49	156.7 (111.6–228.6)	40			1.49	19.42	
IIb	H——$COCH_2C_6H_5$	2.12 ± 0.34	74.1 (45.9–109.5)	60	27.8 (14.4–53.4)	45	27.66	40.85	0.39
IIc	H——D-COCH(CH_3)C_6H_5	0.50 ± 0.04	19.2 (14.2–26.1)	65	39.5 (25.1–55.7)	65	117.3	157.40	0.27
IId	H——CO-Ad	0.31 ± 0.03	10.9 (7.29–14.7)	80	21.7 (16.0–29.4)	65	189.2	276.45	0.50
IIIa	H-Tyr-D-Ala-gPhe-Gly—COH	380.1 ± 19.5	641.5 (482.3–876.4)	35			0.15	4.72	
IIIb	H——$COCH_2C_6H_5$	25.2 ± 2.1	291.7 (231.2–374.5)	45			2.32	10.38	
IIIc	H——D-COCH(CH_3)C_6H_5	31.2 ± 2.1	269.1 (249.6–442.3)	60			1.87	11.26	
IIId	H——CO-Ad	19.2 ± 1.2	245.8 (148.5–406.8)	55			3.04	12.36	
DTa	H-Tyr-D-Ala-Phe-Gly——NH_2	45.2 ± 3.2	68.05 (46.1–120.7)	30	68.12 (28.11–99.7)		1.31	44.55	0.16
DTb	H——$NHCH_2C_6H_5$	2.97 ± 0.5	33.06 (22.4–71.0)	30			18.91	91.81	
DTc	H——D-NHCH(CH_3)C_6H_5	0.59 ± 0.1	7.61 (5.46–10.6)	40	13.62 (8.9–20.1)		99.01	398.16	0.85
DTd	H——NH-Ad	2.05 ± 0.1	5.12 (3.40–12.6)	35	18.79 (13.4–25.6)		39.4	591.79	0.58
DTe	H——OH	108.0 ± 8.2	140.4 (102.2–207.3)	25			0.41	21.61	
DTf	H——OEt	191.2 ± 12.6	492.1 (361.7–734.5)	20			0.44	6.15	
	dermorphin	1.41 ± 0.14	11.39 (6.60–27.05)	40	2.75 (1.73–6.42)	35	41.5	266.02	3.98
	morphine	58.6 ± 4.6	3030 (2207–4162)	65	10.9 (7.02–18.8)	60	1	1	1

[a] The values are the means of six experiments ± SEM. [b] Analgesia of at least four doses of each compound was investigated. Each dose was tested for at least 10 animals. [c] Relative potencies are on molar basis (morphine = 1).

These findings fit the results obtained by other investigators on opioid peptides.[21]

More work on the new compounds synthesized will be published elsewhere.

Experimental Section

Melting points were determined on a Tottoli apparatus in open capillaries and are uncorrected. Optical rotations were determined with a Perkin-Elmer 141 polarimeter with a 10-cm water-jacketed cell. HPLC analysis was performed on a Jasco liquid chromatograph equipped with a Varichrom UV variable-wavelength detector and an M660 solvent programmer. A Waters C-18 μ-Bondapak column (30 cm × 4 mm) was used in the HPLC system. All organic solvents were UV spectroscopic grade and were filtered and degassed prior to use. Analytical determination for each deprotected pseudopeptide was obtained by running a linear, 30-min gradient from 10% acetonitrile–90% 0.01 M ammonium formate, pH 4.0, to 60% acetonitrile–30% 0.01 M ammonium formate at a flow rate of 2 mL/min. The desired pseudopeptides eluted in the range of 20–30% acetonitrile. All DT analogues demonstrated impurities of less than 1% by analytical HPLC with UV monitoring at 210 and 280 nm. The amino acid composition was determined with a Carlo Erba 3A29 amino acid analyzer after acid hydrolysis in constant-boiling HCl containing phenol (1%). Acid hydrolysis of a *gem*-diamine moiety yields 2 mol of ammonia; a malonyl residue is not detectable by amino acid analysis. TLC was performed on precoated plates of silica gel F254 (from E. Merck) with use of the following solvent systems: (A) 1-butanol/AcOH/H_2O (6:1:5), (B) EtOAc/pyridine/AcOH/H_2O (60:20:6:11), (C) $CHCl_3$/MeOH/benzene (85:10:5), (D) $CHCl_3$/MeOH (1:1), (E) $CHCl_3$/AcOH/benzene (85:10:5), (F) EtOAc/MeOH (1:1), (G) $CHCl_3$/MeOH/H_2O (80:20:2). Ninhydrin 1% (Merck), fluorescamine (Hoffman-La Roche), and/or chlorine reagent were used as spray reagents. Samples were considered pure when they showed single spots with more than one solvent systems. Elemental analyses indicated by the symbols of the elements refer to data within ±0.4% of the theoretical values. Analyses were carried out after drying the products for 12 h at 50 °C (0.2 torr). Column chromatography was used (when necessary) for preparative purification of crude products. Open column chromatography was run on silica gel 60 (70–230 mesh, Merck).

Coupling Procedures. Method A. To a stirred solution (0.5–0.8 M) of Boc-protected amino acid or Boc-protected peptide (1 mmol) in DMF, 1 equiv of *N*-methylmorpholine (NMM) was added; the mixture was cooled to −10 °C, treated with isobutyl chloroformate (IBCF) (1 equiv), and allowed to react for 2–3 min. A precooled solution of amino component hydrochloride or trifluoroacetate (1.1 mmol) in DMF (0.4–0.6 M) was added to the mixture, followed by NMM (1.1 equiv). The reaction mixture was stirred for 1 h at −10 °C and 2–3 h at 0–10 °C and then diluted with EtOAc (100 mL). The solution or suspension was washed consecutively with brine, 0.5 N $KHSO_4$, brine, 5% $NaHCO_3$, and brine. The organic phase was dried ($MgSO_4$), filtered and evaporated to dryness. The residue was crystallized from appropriate solvents or further purified by column chromatography.

Method B. To a solution of the carboxy component (2 mmol) in DMF (10 mL) were added the amino component (2 mmol), NMR (2 mmol, only when the amino component was in the protonated form), 1-hydroxybenzotirazole (HOBt) (2 equiv), and DCC (2.1 mmol) in the above order at 0 °C. The reaction mixture

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was stirred for 2 h at 0 °C and for 24 h at room temperature; *N,N'*-dicyclohexylurea (DCU) was filtered off and the solution was diluted with EtOAc (100 mL) and worked up as described in method A.

Deprotection. Method C. Boc protecting groups were removed by treating the peptide with aqueous 90% TFA (1:10, w/v) containing anisole (1 mL) for 30–40 min. The solvent was evaporated in vacuo at 0 °C, and the residue was triturated with Et_2O or petrol ether (PE): the resulting solid peptide was collected and dried.

Method D. Hydrogenations were carried out in the indicated solvent at atmospheric pressure and room temperature, in the presence of 10% palladized charcoal (catalyst to peptide ratio, 1:9, w/w). The reaction mixture was filtered through a Celite bed and evaporated to dryness. The residue was treated as described above in method C.

Preparation of Geminal Diamine Derivatives. To a stirred suspension of Z-protected dipeptide amide (3 mmol) in 10 mL of acetonitrile–water (6:4) was added bis(trifluoroacetoxy)iodobenzene (TIB) (1.01 equiv).[15a] The resulting solution was stirred for 3–4 h under N_2 at room temperature, following the reaction by TLC (solvent system B); the acetonitrile was removed, and the aqueous layer was acidified with 2 N HCl (2 mL) and lyophilized. The resulting diamine hydrochloride was recrystallized from EtOH–Et_2O. The following compounds were obtained.

Z-D-Ala-gPhe-H·HCl (1), from Z-D-Ala-Phe-NH_2,[10a] 81% yield; mp 127–129 °C; $[\alpha]^{25}_D$ +4.9° (*c* 1.0, MeOH). Anal. ($C_{19}H_{24}N_3O_3Cl$) C, H, N.

Z-Phe-gGly-H·HCl (6), from Z-Phe-Gly-NH_2,[4b] 87% yield; mp 146–148 °C; $[\alpha]^{25}_D$ +4.5° (*c* 1.0, MeOH). Anal. ($C_{18}H_{22}N_3O_3Cl$) C, H, N.

Z-D-Ala-gPhe-mGly-OEt (2). To an ice-cold stirred suspension of **1** (0.87 g, 2.3 mmol) in DMF (8 mL), containing TEA (0.64 mL, 4.6 mmol), was added ethyl malonyl chloride (0.36 mL, 2.75 mmol). The recation mixture was stirred at 0–5 °C for 3 h, diluted with EtOAc (100 mL), and then worked up (procedure A). The crude product **2** was recrystallized from EtOAc (0.7 g, 67%): mp 158–160 °C; $[\alpha]^{25}_D$ +39.1° (*c* 1.0, MeOH). Anal. ($C_{24}H_{29}N_3O_6$) C, H, N.

Boc-Tyr-D-Ala-gPhe-mGly-OEt (3). Compound **2** (0.91 g, 2 mmol) in AcOH (30 mL) was hydrogenated for 3 h (procedure D). The resulting H-D-Ala-gPhe-mGly-OEt acetate (0.76 g, 2 mmol) in DMF (15 mL) was treated with TEA (0.28 mL, 2 mmol) and Boc-Tyr-OSu (0.68 g, 1.8 mmol). The reaction mixture was stirred for 6 h at room temperature, diluted with EtOAc (100 mL), and worked up as in method A. The resulting **3** was recrystallized from EtOH–Et_2O (0.84 g, 80%): mp 201–203 °C; $[\alpha]^{25}_D$ +45.2° (*c* 1.0, MeOH); TLC, R_f (C) 0.53; amino acid analysis, Tyr 0.98, Ala 1.00, NH_3 2.01. Anal. ($C_{30}H_{40}N_4O_8$) C, H, N.

Boc-Tyr-D-Ala-gPhe-mGly-OH (4). To a solution of **3** (2.33 g, 4 mmol) in EtOH (18 mL) was added 1 M NaOH (4.4 mL) and the mixture was stirred for 2 h while the progress of the hydrolysis was checked by TLC (solvent system B). After evaporation of the ethanol, the mixture was diluted with water (20 mL) and washed with EtOAc (2 × 25 mL). The precooled aqueous phase was then acidified with solid citric acid and extracted with EtOAc (3 × 30 mL). The organic extract was washed with brine, dried, and evaporated to dryness. The resulting **4** was recrystallized from EtOH (1.9 g, 86%): mp 179–181 °C; $[\alpha]^{25}_D$ +41.3° (*c* 1.0, MeOH); TLC, R_f (B) 0.61; amino acid analysis, Tyr 0.99, Ala 1.00, NH_3 1.96.

Boc-Tyr-D-Ala-gPhe-mGly-NH_2 (5a). According to the general coupling procedure B, **4** was treated with the ammonium salt of HOBt.[22] The crude **5a** was recrystallized from EtOH (73%): mp 124–126 °C; $[\alpha]^{25}_D$ +35.6° (*c* 1.08 MeOH); TLC, R_f (C) 0.51; amino acid analysis, Tyr 0.97, Ala 1.00, NH_3 2.98. Anal. ($C_{28}H_{37}N_5O_7$) C, H, N.

Boc-Tyr-D-Ala-gPhe-mGly-NH-Bzl (5b), from **4** and benzylamine (procedure B). The crude **5b** was purified by flash chromatography[23] on silica gel with use of the solvent system D. It was recrystallized from EtOAc–Et_2O (62%): mp 161–163 °C; $[\alpha]^{25}_D$ +45.0° (*c* 1.0, MeOH); TLC, R_f (C) 0.54. Anal. ($C_{35}H_{43}N_5O_7$) C, H, N.

Boc-Tyr-D-Ala-gPhe-mGly-D-NH-CH(CH_3)C_6H_5 (5c), from **4** and (*R*)-(+)-α-methylbenzylamine (procedure B). Compound **5c** was crystallized from EtOAc (70%): mp 160–162 °C; $[\alpha]^{25}_D$ +55.5° (*c* 1.0, MeOH); TLC, R_f (C) 0.56. Anal. ($C_{36}H_{45}N_5O_7$) C, H, N.

Boc-Tyr-D-Ala-gPhe-mGly-NH-Ad (5d), from **4** and 1-adamantanamine (procedure B). Compound **5d** was recrystallized from EtOAc–PE (71%): mp 133–135 °C; $[\alpha]^{25}_D$ +40.8° (*c* 1.0, MeOH); TLC, R_f (E) 0.71. Anal. ($C_{38}H_{51}N_5O_7$) C, H, N.

Z-Phe-gGly-COH (7a). To a stirred suspension of **6** (3.63 g, 10 mmol) in 98% formic acid (10.5 mL), cooled at 0 °C, was added dropwise acetic anhydride (3.5 mL). After 3 h at 0 °C, crushed ice (50 g) was added. The resulting precipitate was collected, washed with water, and dried (P_2O_5, in vacuo). The crude **7a** was recrystallized from EtOH (3.1 g, 87%): mp 190–191 °C; $[\alpha]^{25}_D$ −11.3° (*c* 1.0, DMF); TLC, R_f (C) 0.61. Anal. ($C_{19}H_{21}N_3O_4$) C, H, N.

Z-Phe-gGly-COBzl (7b). To a suspension of **6** (3.63 g, 10 mmol) in DMF (15 mL) containing TEA (1.4 mL, 10 mmol), was added 1-succinimidylphenyl acetate (2.33 g, 10 mmol).[24] The reaction mixture was stirred for 10 h, diluted with EtOAc (150 mL), and worked up as in coupling procedure A. The resulting **7b** was reprecipitated from EtOH–Et_2O (3.6 g, 80%): mp 208–210 °C; $[\alpha]^{25}_D$ −11.1° (*c* 1.0, DMF). Anal. ($C_{26}H_{27}N_3O_4$) C, H, N.

Z-Phe-gGly-D-COCH(CH_3)C_6H_5 (7c) was prepared by coupling **6** with (*S*)-(+)-2-phenylpropionic acid (procedure B). It was recrystallized from EtOAc (82%): mp 210–212 °C; TLC, R_f (C) 0.61. Anal. ($C_{27}H_{29}N_3O_4$) C, H, N.

Z-Phe-gGly-CO-Ad (7d), from **6** and 1-adamantanecarbonyl chloride as described for **2**. Compound **7d** was recrystallized from EtOAc–PE (86%): mp 225–227 °C; $[\alpha]^{25}_D$ +4.0° (*c* 1.0, acetic acid). Anal. ($C_{29}H_{35}N_3O_4$) C, H, N.

Boc-Tyr-D-Ala-Phe-gGly-COH (9a). Compound **7a** (0.39 g, 1.1 mmol) in AcOH–H_2O (13:2) was hydrogenated for 2 h (procedure D). The resulting H-Phe-gGly-COH acetate (0.3 g) in DMF (8 mL) was treated with Boc-Tyr-D-Ala-OH[4b] (**8**; 0.35 g, 1 mmol) (coupling procedure B). It was recrystallized from EtOAc (0.41 g, 72%): mp 120–122 °C; $[\alpha]^{25}_D$ +6.9° (*c* 1.0, MeOH); TLC, R_f (E) 0.56; amino acid analysis, Tyr 0.97, Ala 1.01, Phe 1.00, NH_3 1.97. Anal. ($C_{28}H_{37}N_5O_7$) C, H, N.

Boc-Tyr-D-Ala-Phe-gGly-CO-Bzl (9b) was prepared as **9a** from **8** and H-Phe-gGly-CO-Bzl acetate (obtained from **7b** following procedure D). It was recrystallized from EtOH–Et_2O (73%): mp 179–181 °C; $[\alpha]^{25}_D$ +15.0° (*c* 1.0, MeOH); amino acid analysis, Tyr 0.96, Ala 1.00, Phe 1.02, NH_3 1.95.

Boc-Tyr-D-Ala-Phe-gGly-D-COCH(CH_3)C_6H_5 (9c), from **8** and H-Phe-gGly-D-COCH(CH_3)C_6H_5 (procedure B). Compound **9c** was recrystallized from EtOAc (76%): mp 146–148 °C; $[\alpha]^{25}_D$ +14.7° (*c* 1.0, MeOH); TLC, R_f (E) 0.61. Anal. ($C_{36}H_{45}N_5O_7$) C, H, N.

Boc-Tyr-D-Ala-Phe-gGly-CO-Ad (9d) from **8** and H-Phe-gGly-CO-Ad acetate (procedure B). Compound **9d** was crystallized from EtOAc–Et_2O (70%): mp 140–142 °C; $[\alpha]^{25}_D$ +7.6° (*c* 1.0, MeOH); TLC, R_f (C) 0.73. Anal. ($C_{38}H_{51}N_5O_7$) C, H, N.

***N*-(Phenylacetyl)glycine (10b).** Glycine (0.75 g, 10 mmol) in 2 N NaOH (10 mL) was treated with 1-succinimidyl phenylacetate (1.16 g, 5 mmol) in THF (10 mL) with stirring at 0 °C. The reaction mixture was left at 0–5 °C for 6 h, and THF was removed under reduced pressure. The aqueous solution was adjusted to pH 1 with 5 N HCl and extracted with EtOAc (2 × 20 mL). The combined organic extracts were dried, filtered, and evaporated to dryness, and the residue was recrystallized from EtOAc (0.8 g, 83%): mp 140–142 °C; TLC, R_f (C) 0.58. Anal. ($C_{10}H_{11}NO_3$) C, H, N.

***N*-[(*S*)-2-Phenylpropionyl]glycine (10c)**, from glycine and 1-succinimidyl (*S*)-2-phenylpropionate (1.23 g, 5 mmol), as **10b**. It was reprecipitated from EtOAc–PE (0.78 g, 75%): mp 97–99 °C; TLC, R_f (C) 0.60. Anal. ($C_{11}H_{13}O_3N$) C, H, N.

***N*-(1-Adamantylcarbonyl)glycine (10d)**, from glycine and 1-adamantanecarbonyl chloride (0.99 g, 5 mmol) as **10b**. The title compound was recrystallized from EtOH (1 g, 84%): mp 194–196

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°C; TLC, R_f (C) 0.66. Anal. ($C_{13}H_{19}NO_3$) C, H, N.

Z-D-Ala-gPhe-Gly-COH (11a). OHC-Gly-OH (0.103 g, 1 mmol) in DMF (8 mL) was treated with 1 (Z-D-Ala-gPhe-H·HCl) (0.414 g, 1.1 mmol) in the presence of TEA (0.14 ml, 1 mmol) via DCC/HOBt (1 equiv) (procedure B). The pseudotripeptide **11a** was recrystallized from MeOH (0.35 g, 83%): mp 219–221 °C; $[\alpha]^{25}_D$ +11.1° (*c* 1.0, DMF); TLC, R_f (C) 0.56. Anal. ($C_{22}H_{26}N_4O_5$) C, H, N.

Z-D-Ala-gPhe-Gly-CO-Bzl (11b), from *N*-(phenylacetyl)-glycine and 1 (procedure B). Compound **11b** was recrystallized from EtOAc (74%): mp 219–220 °C; $[\alpha]^{25}_D$ +8.5° (*c* 1.0, DMF); amino acid analysis, Ala 1.01, Gly 1.00, NH_3 1.96. Anal. ($C_{29}H_{32}N_4O_5$) C, H, N.

Z-D-Ala-gPhe-Gly-D-COCH(CH$_3$)C$_6$H$_5$ (11c), from *N*-[(*S*)-(+)-2-phenylpropionyl]glycine and 1 (procedure B). Compound **11c** was recrystallized from EtOH (71%): mp 188–189 °C; $[\alpha]^{25}_D$ +12.7° (*c* 1.0, DMF). Anal. ($C_{30}H_{34}N_4O_5$) C, H, N.

Z-D-Ala-gPhe-Gly-CO-Ad (11d), from *N*-(1-adamantylcarboxy)glycine and 1 (procedure B). Compound **11d** was recrystallized from EtOAc (69%): mp 103–105 °C; $[\alpha]^{25}_D$ +21.4° (*c*, 1.0, acetic acid); TLC, R_f (C) 0.77. Anal. ($C_{32}H_{40}N_4O_5$) C, H, N.

Boc-Tyr-D-Ala-gPhe-Gly-COH (12a). A sample of **11a** (0.468 g, 1.1 mmol) in AcOH (15 mL) was hydrogenated for 3 h (procedure D). The resulting H-D-Ala-gPhe-Gly-COH acetate (0.387 g, 1.1 mmol) in DMF (8 mL) containing TEA (0.154 mL, 1.1 mmol) was treated with Boc-Tyr-OSu (0.37 g, 1 mmol). The reaction mixture was stirred for 8 h at room temperature, diluted with EtOAc (100 mL), and worked up as in procedure A. The crude product was recrystallized from EtOAc (82%): mp 158–160 °C; $[\alpha]^{25}_D$ +21.6° (*c* 1.0, acetic acid); TLC, R_f (C) 0.61; amino acid analysis, Tyr 0.96, Ala 1.01, Gly 1.00, NH_3 1.94. Anal. ($C_{28}H_{37}N_5O_7$) C, H, N.

Boc-Tyr-D-Ala-gPhe-Gly-CO-Bzl (12b). Compound **11b** was hydrogenated for 3 h (procedure D). The resulting H-D-Ala-gPhe-Gly-CO-Bzl acetate was treated with Boc-Tyr-OSu as for **12a**. The crude **12b** was recrystallized from EtOH (79%): mp 158–160 °C; $[\alpha]^{25}_D$ +41.2° (*c* 1.0, MeOH); TLC, R_f (C) 0.65. Anal. ($C_{35}H_{43}N_5O_7$) C, H, N.

Boc-Tyr-D-Ala-gPhe-Gly-D-CO-CH(CH$_3$)C$_6$H$_5$ (12c), from H-D-Ala-gPhe-Gly-D-CO-CH(CH_3)C_6H_5 and Boc-Tyr-OSu. Compound **12c** was recrystallized from EtOAc (81%): mp 146–148 °C; $[\alpha]^{25}_D$ +37.7° (*c* 1.0, MeOH). Anal. ($C_{36}H_{45}N_5O_7$) C, H, N.

Boc-Tyr-D-Ala-gPhe-Gly-CO-Ad (12d), from H-D-Ala-gPhe-Gly-CO-Ad and Boc-Tyr-OSu. The title compound was recrystallized from EtOH–H_2O (83%): mp 152–154 °C: $[\alpha]^{25}_D$ +38.6° (*c* 1.0, MeOH); amino acid analysis, Tyr 0.99, Ala 1.04, Gly 1.00, NH_3 1.98. Anal. ($C_{38}H_{51}N_5O_7$) C, H, N.

Preparations of Free Pseudotetrapeptides Ia–f, IIa–d, and IIIa–d. Each Boc-pseudotetrapeptide analogue was deprotected according to procedure C. The resulting free pseudopeptide (1 mmol) was dissolved in 0.5 N acetic acid (2 mL) and passed through a 2 × 40 cm Sephadex G-25 column, using system A as eluting solvent. Evaporation of solvent from the appropriate combined fractions gave the pseudotetrapeptide trifluoroacetates: Ic–d, IId, and IIIa,d were recrystallized from MeOH–Et_2O (80–85%). All other pseudopeptide trifluoroacetates were converted into corresponding acetate salts through Whatman DE52 anion-exchange resin (acetate form) with 0.2 N acetic acid as eluting solvent. The fractions containing the peptide were collected and lyophilized to constant weight (85–90%). The analogue Ie was obtained as a free base. Characterizations of the final 14 analogues are summarized in Table I.

Pharmacological Assays. All pseudopeptides and reference compounds were assayed on the electrically stimulated guinea pig ileum (GPI) with use of the conditions of Kosterlitz and Watt.[16] Dose–response curves were drawn on at least three points and the IC_{50}, i.e., the concentration of compound necessary to inhibit the amplitude of elecrically induced twitch by 50%, was determined. All compounds completely inhibited the twitch at the highest doses. The analgesic potency of pseudopeptides was estimated in Swiss-Webster mice wighing 27–30 g. The tail-flick test was essentially that described by Janssen,[17] using water at 55 °C as nociceptive stimulus. Tests were made prior to and at various times after icv and sc administration of each compound in saline (4 μL). The average reaction time in control animals was 1 s. Complete analgesia was assumed to be present when no reaction appeared up to 10 s after application of noxious stimulus. Percent analgesia was calculated according to the formula $[(T - T_0)/(10 - T_0)] \times 100$. ED_{50} values and the 95% confidence limits were calculated by the methods of Tyers[25] and Finney.[26]

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Registry No. 1, 95617-40-6; 2, 95617-41-7; 3, 95617-42-8; 4, 95617-43-9; **5a**, 95646-79-0; **5b**, 95617-44-0; **5c**, 95617-45-1; **5d**, 95617-46-2; 6, 95617-47-3; **7a**, 95617-48-4; **7b**, 95646-80-3; **7c**, 95617-49-5; **7d**, 95617-50-8; 8, 71591-34-9; **9a**, 95617-51-9; **9b**, 95617-52-0; **9c**, 95617-53-1; **9d**, 95617-54-2; **10a**, 2491-15-8; **10b**, 500-98-1; **10c**, 95721-31-6; **10d**, 21241-41-8; **11a**, 95617-55-3; **11b**, 95617-56-4; **11c**, 95617-57-5; **11d**, 95617-58-6; **12a**, 95617-59-7; **12b**, 95617-60-0; **12c**, 95617-61-1; **12d**, 95617-62-2; Ia·acetate, 95617-64-4; Ia, 95617-63-3; Ib·acetate, 95617-66-6; Ib, 95617-65-5; Ic·trifluoroacetate, 95617-68-8; Ic, 95617-67-7; Id trifluoroacetate, 95722-40-0; Id, 95617-69-9; Ie, 95617-70-2; If·acetate, 95617-72-4; If, 95617-71-3; IIa·acetate, 95617-74-6; IIa, 95617-73-5; IIb·acetate, 95617-76-8; IIb, 95617-75-7; IIc·acetate, 95617-78-0; IIc, 95617-77-9; IId·trifluoroacetate, 95617-80-4; IId, 95617-79-1; IIIa·trifluoroacetate, 95617-82-6; IIIa, 95617-81-5; IIIb·acetate, 95617-84-8; IIIb, 95617-83-7; IIIc·acetate, 95617-86-0; IIIc, 95617-85-9; IIId·trifluoroacetate, 95617-88-2; IIId, 95617-87-1; DTa, 78700-75-1; DTb, 83579-03-7; DTc, 83603-32-1; DTd, 83579-08-2; DTe, 78700-74-0; DTf, 82793-66-6; Z-D-Ala-Phe-NH_2, 95617-89-3; Z-Phe-Gly-NH_2, 17187-05-2; H-D-Ala-Phe-mGly-OEt, 95617-90-6; Boc-Tyr-OSu, 20866-56-2; H-Phe-gGly-COH·acetate, 95646-82-5; H-Phe-gGly-CO-Bzl·acetate, 95646-84-7; H-Phe-gGly-DCOCH(CH_3)C_6H_5, 95617-91-7; H-Phe-gGly-CO-Ad·acetate, 95617-93-9; H-D-Ala-gPhe-CO-CH_2-NH-COH·acetate, 95617-95-1; H-D-Ala-gPhe-CO-CH_2-NH-CO-Bzl·acetate, 95617-97-3; H-D-Ala-gPhe-CO-CH_2-NH-D-CO-CH(CH_3)C_6H_5, 95617-98-4; H-D-Ala-gPhe-CO-CH_2-NH-CO-Ad, 95617-99-5; $ClCOCH_2COOEt$, 36239-09-5; H_2NBzl, 100-46-9; (*R*)-(+)-H_2N-CH(CH_3)C_6H_5, 3886-69-9; H_2NAd, 768-94-5; formic acid, 64-18-6; 1-succinimidyl phenylacetate, 23776-85-4; (*S*)-(+)-2-phenylpropionic acid, 7782-24-3; 1-adamantanecarbonyl chloride, 2094-72-6; glycine, 56-40-6; 1-succinimidyl (*S*)-2-phenylpropionate, 95618-00-1.

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