Opioid Agonists and Antagonists. Peptides Containing *N*-Terminal Allyl Groups and/or a Thiomethylene Linkage in Place of a Peptide Bond

Gianfranco Balboni, Severo Salvadori, Mauro Marastoni, and Roberto Tomatis *
Department of Pharmaceutical Sciences, University of Ferrara, 44100 Ferrara, Italy
Pier A. Borea and Clementina Bianchi
Institute of Pharmacology, University of Ferrara, Italy

Peptides containing *N*-allyl or *N,N*-diallyl groups at the *N*-terminus have been synthesized as potential opioid antagonists. A number of analogues with an amide bond replaced by a thiomethylene group have also been prepared. In brain binding assays and in guinea-pig ileum and mouse vas deferens preparations, the analogues generally displayed low affinity for μ- and δ-receptors as well as agonist activities in *in vitro* tests. *N,N*-Diallyl-Phe-D-Ala-Phe-Gly-NH_2 (**28**) was found to be a moderately potent but highly selective antagonist at δ opiate receptors.

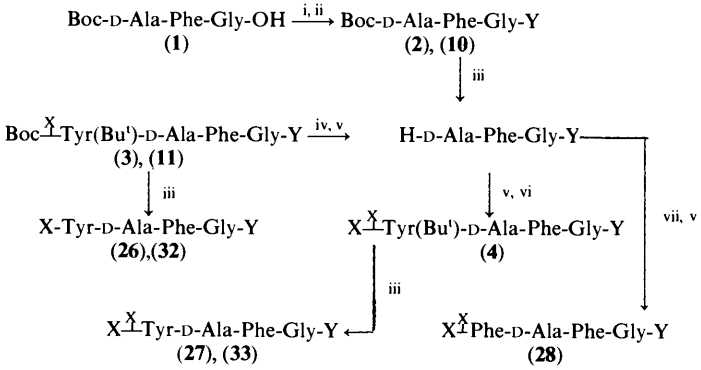
The substitution of a larger moiety such as an allyl, cyclopropylmethyl, or furylmethyl group for the *N*-methyl groups on a non-peptide opioid is known to yield antagonist congeners.[1] Thus substitution of an allyl for the *N*-hydrogen on the Tyr[1] of dermorphin, H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2,[2] or its synthetic analogues is expected to produce antagonists. It has been shown, however, that the *N*-monoallyl derivative of enkephalins is a weak mixed agonist-antagonist,[3,4] while some *N,N*-diallyl-enkephalin analogues exhibit a selective antagonism at the δ-receptor.[5–7] We have previously studied the effect of modifications to the dermorphin structure on both potency and selectivity.[8–13] From this work, it is clear that substitution, particularly at residue 5, of the dermorphin-(1-5) structure produces profound changes in selectivity.[13]

In the present investigation, *N*-allyl and *N,N*-diallyl derivatives of a number of μ- or δ-receptor selective peptide agonists were synthesized and their effects were estimated by bio- and binding assays. Analogues containing a thiomethylene linkage in place of enzymatic biolabile peptide bonds[12] were also prepared and tested.

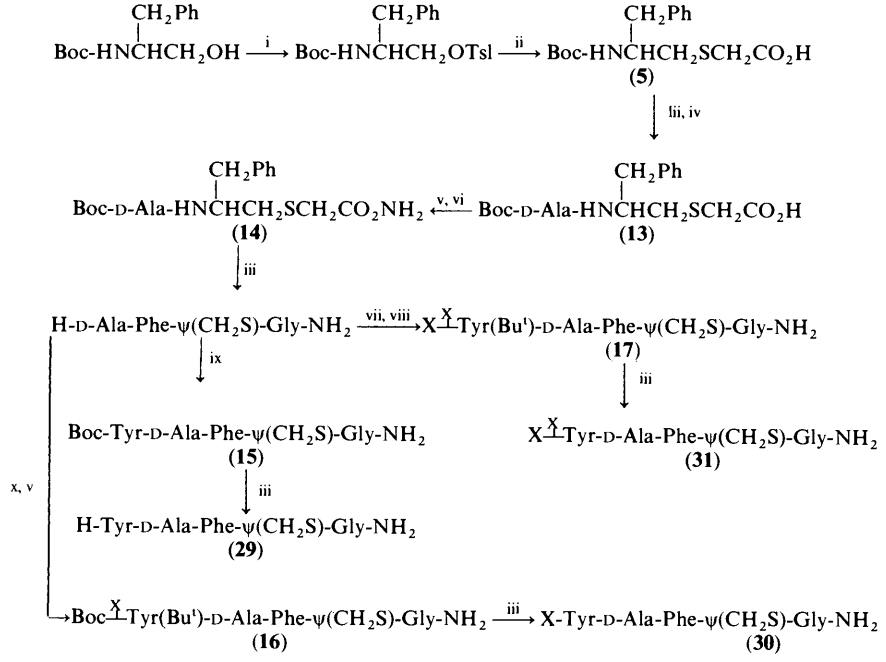
In order to make the structure-activity relationships more meaningful, the opioid data of original dermorphin tetra- and

Table 1. Structures and biological activity of synthetic opioid peptides

		Binding		Agonist activity		Antagonist activity (K_e/nM)	
		[^{3}H]DAGO(μ)	[^{3}H]DADLE(δ)	GPI	MVD		
No.	Compd.	IC_{50}/nM		IC_{50}/nM		*vs.* morphine	*vs.* DADLE
(**26a**)	H-Tyr-D-Ala-Phe-Gly-NH_2	45.7 ± 7	1 438 ± 128	43.6 ± 5	510 ø 63		
(**26**)	Allyl-Tyr-D-Ala-Phe-Gly-NH_2	1 400 ± 190	> 10 000	2 400 ± 300	> 50 000		
(**27**)	$(Allyl)_2$-Tyr-D-Ala-Phe-Gly-NH_2	1 900 ± 300	> 10 000	2 500 ± 300	> 50 000		
(**28**)	$(Allyl)_2$Phe-D-Ala-Phe-Gly-NH_2	> 10 000	10 000	> 10 000	> 50 000	> 5 000	199 ± 68
(**29**)	H-Tyr-D-Ala-Phe-ψ(CH_2S)-Gly-NH_2	110 ± 26	2 500 ± 500	135 ± 15	> 50 000		
(**30**)	Allyl-Tyr-D-Ala-Phe-ψ(CH_2S)-Gly-NH_2	2 400 ± 350	> 10 000	10 000	> 50 000		
(**31**)	$(Allyl)_2$-Tyr-D-Ala-Phe-ψ(CH_2S)-Gly-NH_2	> 10 000	> 10 000	> 10 000	> 50 000		
(**32a**)	H-Tyr-D-Ala-Phe-Gly-Phe- NH_2	26.5 ± 3	626 ± 76	2.3 ± 0.3	19.4 ± 3		
(**32**)	Allyl-Tyr-D-Ala-Phe-Gly-Phe-NH_2	430 ± 70	3 800 ± 600	22 ± 3	940 ± 160		
(**33**)	$(Allyl)_2$-Tyr-D-Ala-Phe-Gly-Phe-NH_2	540 ± 90	4 500 ± 900	315 ± 60	> 50 000		
(**34**)	Allyl-Tyr-D-Ala-Phe-Gly-ψ(CH_2S)-Phe-NH_2	910 ± 150	6 300 ± 1 000	1 050 ± 150	> 50 000		
(**35**)	$(Allyl)_2$-Tyr-D-Ala-Phe-Gly-ψ(CH_2S)-Phe-NH_2	1 480 ± 200	9 800	934 ± 150	> 50 000		
(**36a**)	H-Tyr-D-Ala-Phe-Gly-D-Phgly-NH_2	149 ± 30	43.6 ± 10	37 ± 4	2.3 ± 0.2		
(**36**)	$(Allyl)_2$-Tyr-D-Ala-Phe-Gly-ψ(CH_2S)-D-Phgly-NH_2	9 800	> 10 000	1 450 ± 180	20 000		
	$(Allyl)_2$-Tyr-Gly-Gly-ψ(CH_2S)-Phe-Leu-OH (ICI 154129)	> 10 000	3 500 ± 500	> 10 000	> 50 000	> 5 000	177 ± 24
	H-Tyr-D-Ala-Gly-Phe-D-Leu-OH (DADLE)				1.3 ± 0.2		



Scheme 1. Synthesis of tetra- and penta-peptides containing *N*-allyl- or *N*,*N*-diallyl-tyrosine. *Reagents:* i, $ClCO_2CH_2CH(CH_3)_2$; ii, *N*-methylmorpholine; iii, CF_3CO_2H; iv, Boc-X-Tyr(Bu^t)OH; v, DCCI–HOBt; vi, X-Tyr(Bu^t)OH; vii, X-Phe-OH; X = $CH_2CH{=}CH_2$; for compounds (**2**), (**3**), (**4**), and (**26**)—(**28**), Y = NH_2; for compounds (**10**), (**11**), (**32**), and (**33**), Y = Phe-NH_2.



Scheme 2. Synthesis of tetrapeptide analogues with the amide bond between the 3 and 4 positions replaced by a CH_2S group. *Reagents:* i, *p*-$MeC_6H_4SO_2Cl$ in pyridine; ii, $NaSCH_2CO_2Na$; iii, TFA; iv, Boc-D-Ala-OSu; v, MA; vi, NH_3; vii, X-Tyr(Bu^t)OH; viii, DCCI–HOBt; ix, Boc-Tyr-OSu; x, Boc-X-Tyr(Bu^t)OH; X = $CH_2CH{=}CH_2$

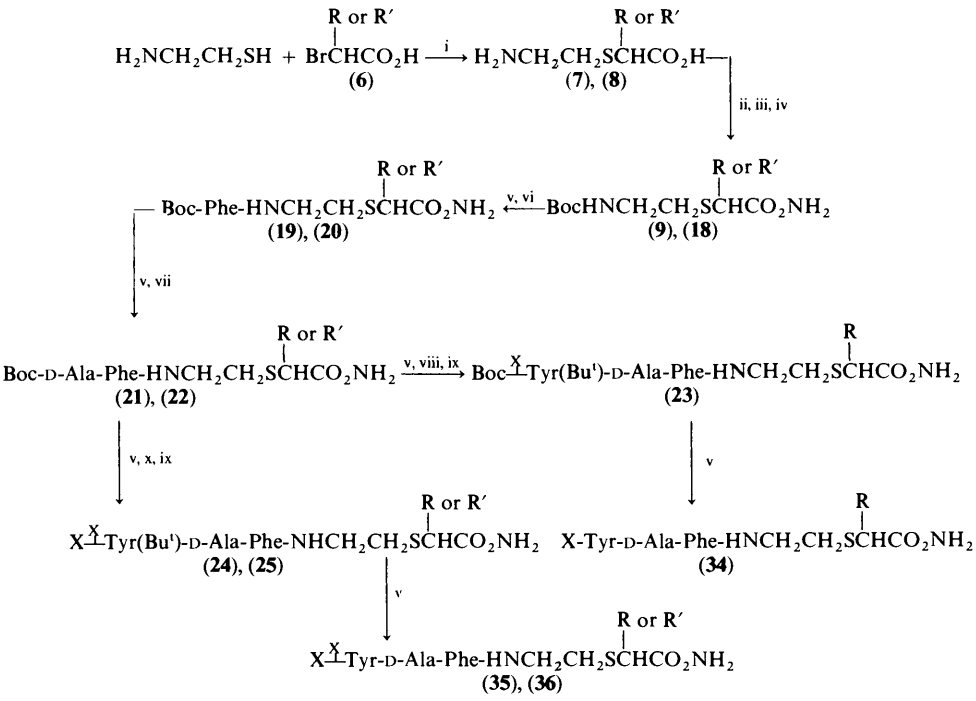
penta-peptides[9,13] (**26a**), (**32a**), and (**36a**) and the δ-receptor antagonist *N*,*N*-diallyl-Tyr-Gly-Gly-ψ(CH_2S)-Phe-Leu-OH (ICI 154129)[5] are also reported (Table 1).

Synthesis.—All the peptides reported here are listed in Table 1. *N*-Allyl- or *N*,*N*-diallyl-(Tyr-D-Ala-Phe-Gly-Y analogues [(**26**)—(**28**), Y = NH_2] and [(**32**) and (**33**), Y = Phe-NH_2] were prepared by the route shown in Scheme 1.

Modifications only at *N*- and *C*-termini suggested a route in which the segment H-D-Ala-Phe-Gly-Y is first prepared and is subsequently condensed with the appropriate tyrosine derivative, yielding protected tetra- or penta-peptides. The removal of the protecting groups, Boc and/or Bu^t, by trifluoroacetic acid gave the expected *N*-monoallyl-(**26**), (**32**), and *N*,*N*-diallyl derivatives (**27**), (**28**), and (**33**).

The analogues containing a CH_2S linkage in place of an amide bond between Phe[3]-Gly[4] were synthesized according to the route shown in Scheme 2.

3-Phenyl-2-t-butoxycarbonylaminopropan-1-ol (Boc-phenylalaninol) was tosylated by reaction with toluene-*p*-sulphonyl chloride in pyridine. The sulphonate was then treated with disodium mercaptoacetate and the resulting 2-(3-phenyl-2-t-butoxycarbonylaminopropylthio)acetic acid [Boc-Phe-ψ(CH_2S)-Gly-OH] was converted into an amide derivative by the mixed anhydride method. Deprotection, followed by coupling with the appropriate Boc-amino acids yielded the fully protected pseudotetrapeptides. Subsequent removal of protecting groups by trifluoroacetic acid gave the desired analogues (**29**)—(**31**). The pseudopentapeptides (**34**)—(**36**) containing a CH_2S linkage between positions 4 and 5 were prepared by the



Scheme 3. Synthesis of pentapeptide analogues with the amide bond between the 4 and 5 positions replaced by a CH_2S group. *Reagents:* i, $NaHCO_3$; ii, $(Boc)_2O$; iii, MA; iv, NH_3; v, TFA; vi, Boc-Phe-OH; vii, Boc-D-Ala-OSu; viii, Boc-X-Tyr(But)OH; ix, DCCI-HOBt; x, X-X-Tyr(But)OH; for compounds (**7**), (**9**), (**19**), (**21**), (**23**), and (**24**), R = CH_2Ph; for compounds (**8**), (**18**), (**20**), (**22**), and (**25**), R′ = Ph; X = $CH_2CH{=}CH_2$

route shown in Scheme 3. The procedures used for the synthesis of 2-(2-aminoethylthio)alkanoic acid derivatives were based on the routes reported earlier for these thiopeptides.[14,15]

(*R*)-2-Bromo-3-phenylpropionic acid and (*S*)-2-bromo-2-phenylacetic acid were prepared from D-phenylalanine and L-phenylglycine respectively by treatment with sodium nitrite and potassium bromide in aqueous sulphuric acid. This reaction has been shown to proceed with retention of configuration. On the other hand, since the reaction of thiol derivatives with bromo acids is known to proceed with inversion of configuration, (*S*)-2-(2-aminoethylthio)-3-phenylpropionic acid [Gly-ψ-(CH_2S)-L-Phe] and (*R*)-2-(2-aminoethylthio)-2-phenylacetic acid [Gly-ψ(CH_2S)-D-Phgly] were obtained starting from D-phenylalanine and L-phenylglycine respectively. The final thiopentapeptide analogues (**34**)—(**36**) were obtained using active esters, mixed anhydrides or the DCCI–HOBt method,[16] through the stepwise addition of the appropriate amino acid to the pseudodipeptide units as depicted in Scheme 3.

Bioassays and Binding Assays.—All the analogues listed in Table 1 were examined for their ability to inhibit the electrically induced contractions of guinea-pig ileum[17] (GPI) and mouse vas deferens[18] (MVD). Relative opioid receptor affinities were determined by the displacement of selective radioligands from guinea-pig brain membrane preparations.[19] [^{3}H][D-Ala^2, MePhe^4, Gly-ol^5]enkephalin (DAGO) served as a highly selective μ-receptor radioligand, and the somewhat less selective radiolabelled [^{3}H][D-Ala^2, D-Leu^5]enkephalin (DADLE) and [^{3}H](±)ethylketazocine (EKC) were used for determining relative δ- and κ-receptor affinities, respectively. Antagonist activity was measured in the field-stimulated GPI and MVD preparations. K_e Values[20,*] were determined against morphine and DADLE. All results represent the mean of at least four determinations (Table 1).

* Negative log of molar concentration that reduces agonist activity by 50%.

Results and Discussion

As previously observed for various series of dermorphin peptides,[12] none of the present analogues displaced the binding of [^{3}H]EKC to any significant degree. The high IC_{50} values (> 10μM, data not shown) indicate that they have a negligible affinity for the κ-binding site.

In the [^{3}H]DAGO and [^{3}H]DADLE binding assays, with the exception of compound (**28**), all the new analogues (**26**)—(**36**) displayed a preference for μ- over δ-receptors but, in comparison to the parent peptides (**26a**), (**32a**), and (**36a**), they showed a lower affinity for both μ- and δ-sites with an evident dependence on *N*-terminal and/or peptide bond modifications.

The results obtained with compounds (**26**)—(**36**) in the GPI and MVD preparations are generally in agreement with the binding data. Whereas the substitution of CH_2S for an amide bond was relatively tolerated [*e.g.*, compound (**26a**) *vs.* (**29**)], *N*-terminal allylation [derivatives (**26**), (**30**), and (**32**)] significantly reduced the relative agonist potency. Compared with these mono-substituted analogues, lower activities were obtained with *N*,*N*-diallyl peptides (**27**), (**31**), and (**33**)—(**36**). Finally, in comparison with the potent and relatively δ-selective agonist (**36a**), its *N*,*N*-diallyl analogue (**36**) was a weak μ-partial agonist, showing an opposite activity pattern.

Among the new compounds synthesized, only *N*,*N*-diallyl-Phe-D-Ala-Phe-Gly-NH_2 (**28**) had antagonistic properties; it seemed to be a moderately potent but highly selective δ-antagonist. In fact, the results on the MVD and GPI assays show that compound (**28**) is as potent as *N*,*N*-diallyl-Tyr-Gly-Gly-ψ(CH_2S)-Phe-Leu-OH (similar K_e values) in antagonising the δ-receptor agonist DADLE, while it is inactive against the μ-agonist morphine. Taken together, the data of the present

Table 2. Physicochemical and analytical data for peptides (**10**)—(**36**)

No.	Compound	Yield (%)	M.p. (°C)	$[\alpha]_D^{24}$ [a]	R_F [b]	Formula	Found (%) (Required) C	H	N	S
(**10**)	Boc-D-Ala-Phe-Gly-Phe-NH_2	73	179—181	+6.4	0.56(C)	$C_{28}H_{37}N_5O_6$	62.2 (62.3)	6.8 (6.9)	13.1 (13.0)	
(**11**)	Boc-(*N*-allyl)Tyr(Bu^t)-D-Ala-Phe-Gly-Phe-NH_2	82	84—86	−5.1	0.61(D)	$C_{44}H_{58}N_6O_8$	66.3 (66.1)	7.2 (7.3)	10.6 (10.5)	
(**12**)	$(Allyl)_2$Tyr(Bu^t)-D-Ala-Phe-Gly-Phe-NH_2	84	98—100	−3.0	0.59(D)	$C_{42}H_{54}N_6O_6$	68.2 (68.3)	7.4 (7.4)	11.5 (11.4)	
(**13**)	Boc-D-Ala-Phe-ψ(CH_2S)Gly-OH	65	Oil	+43.2	0.64(B)	$C_{19}H_{28}N_2O_5S$	57.7 (57.5)	7.2 (7.1)	7.0 (7.1)	8.1 (8.0)
(**14**)	Boc-D-Ala-Phe-ψ(CH_2S)-Gly-NH_2	72	Oil	+42.7	0.67(C)	$C_{19}H_{29}N_3O_4S$	57.5 (57.7)	7.5 (7.4)	10.7 (10.6)	
(**15**)	Boc-Tyr-D-Ala-Phe-ψ(CH_2S)-Gly-NH_2	68	Oil	+40.7	0.78(A)	$C_{28}H_{38}N_4O_6S$	60.4 (60.2)	6.8 (6.9)	10.1 (10.0)	5.8 (5.7)
(**16**)	Boc(*N*-allyl)Tyr(Bu^t)-D-Ala-Phe-ψ(CH_2S)-Gly-NH_2	66	Oil	−31.1	0.68(C)	$C_{35}H_{50}N_4O_6S$	64.1 (64.2)	7.8 (7.7)	8.6 (8.5)	
(**17**)	$(Allyl)_2$Tyr(Bu^t)-D-Ala-Phe-ψ(CH_2S)-Gly-NH_2	71	Oil	+13.7	0.57(C)	$C_{33}H_{46}N_4O_4S$	63.1 (63.2)	7.4 (7.4)	8.9 (8.9)	5.2 (5.1)
(**18**)	Boc-Gly-ψ(CH_2S)-D-Phgly-NH_2	48	89—91	−9.3	0.50(C)	$C_{15}H_{22}N_2O_3S$	57.9 (58.0)	7.2 (7.1)	9.1 (9.0)	
(**19**)	Boc-Phe-Gly-ψ(CH_2S)-Phe-NH_2	77	116—118	−20.7	0.66(C)	$C_{25}H_{33}N_3O_4S$	63.6 (63.7)	7.1 (7.0)	8.9 (8.9)	6.9 (6.8)
(**20**)	Boc(Phe-Gly-ψ(CH_2S)-D-Phgly-NH_2	81	68—70	−13.4	0.64(C)	$C_{24}H_{31}N_3O_4S$	62.9 (63.0)	6.9 (6.8)	9.3 (9.2)	7.0 (6.9)
(**21**)	Boc-D-Ala-Phe-Gly-ψ(CH_2S)-Phe-NH_2	78	73—75	−17.3	0.51(C)	$C_{28}H_{38}N_4O_5S$	61.7 (61.9)	7.0 (7.1)	10.4 (10.3)	
(**22**)	Boc-D-Ala-Phe-Gly-ψ(CH_2S)-D-Phgly-NH_2	72	71—73	−4.3	0.53(C)	$C_{27}H_{36}N_4O_5S$	61.1 (61.3)	6.8 (6.8)	10.5 (10.6)	6.1 (6.0)
(**23**)	Boc(*N*-allyl)Tyr(Bu^t)-D-Ala-Phe-Gly-ψ(CH_2S)-Phe-NH_2	84	46—48	−9.1	0.46(C)	$C_{44}H_{39}N_5O_7S$	67.4 (67.6)	5.1 (5.0)	8.8 (8.9)	4.2 (4.1)
(**24**)	$(Allyl)_2$Tyr(Bu^t)-D-Ala-Phe-Gly-ψ(CH_2S)-Phe-NH_2	82	100—102	−7.3	0.60(C)	$C_{42}H_{55}N_5O_5S$	67.7 (67.9)	7.4 (7.5)	9.5 (9.4)	4.4 (4.3)
(**25**)	$(Allyl)_2$Tyr(Bu^t)-D-Ala-Phe-Gly-ψ(CH_2S)-D-Phgly-NH_2	78	94—96	−3.1	0.61(C)	$C_{41}H_{53}N_5O_5S$	67.4 (67.5)	7.2 (7.3)	9.7 (9.6)	4.5 (4.4)
(**26**)	Allyl-Tyr-D-Ala-Phe-Gly-NH_2	89	115—118	+13.2	0.51(B)	$C_{26}H_{33}N_5O_5C_2HF_3O_2$	55.1 (55.2)	5.7 (5.6)	11.6 (11.5)	
(**27**)	$(Allyl)_2$-Tyr-D-Ala-Phe-Gly-NH_2	93	86—88	−4.7	0.56(A)	$C_{29}H_{37}N_5O_5C_2HF_3O_2$	57.6 (57.3)	6.0 (5.9)	10.9 (10.8)	
(**28**)	$(Allyl)_2$-Phe-D-Ala-Phe-Gly-NH_2	88	155—157	+21.6	0.53(D)	$C_{29}H_{37}N_5O_4C_2HF_3O_2$	58.6 (58.7)	6.1 (6.0)	11.1 (11.0)	
(**29**)	H-Tyr-D-Ala-Phe-ψ(CH_2S)-Gly-NH_2	93	105—107	+52.7	0.48(B)	$C_{23}H_{30}N_4O_4SC_2HF_3O_2$	52.3 (52.4)	5.3 (5.4)	9.9 (9.8)	5.7 (5.6)
(**30**)	Allyl-Tyr-D-Ala-Phe-ψ(CH_2S)-Gly-NH_2	91	85—87	+58.2	0.56(B)	$C_{26}H_{34}N_4O_4SC_2HF_3O_2$	54.7 (54.9)	5.8 (5.7)	9.2 (9.1)	5.3 (5.2)
(**31**)	$(Allyl)_2$-Tyr-D-Ala-Phe-ψ(CH_2S)-Gly-NH_2	89	Oil	+24.3	0.48(B)	$C_{29}H_{38}N_4O_4SC_2HF_3O_2$	57.3 (57.1)	6.1 (6.0)	8.5 (8.6)	5.0 (4.9)
(**32**)	Allyl-Tyr-D-Ala-Phe-Gly-Phe-NH_2	88	104 107	+7.6	0.34(D)	$C_{35}H_{44}N_6O_6C_2HF_3O_2$	58.6 (58.7)	5.8 (5.7)	11.2 (11.1	
(**33**)	$(Allyl)_2$-Tyr-D-Ala-Phe-Gly-Phe-NH_2	91	118—120	+16.7	0.59(A)	$C_{38}H_{46}N_6O_6C_2HF_3O_2$	60.0 (60.3)	6.0 (5.8)	10.7 (10.5)	
(**34**)	Allyl-Tyr-D-Ala-Phe-Gly-ψ(CH_2S)-Phe-NH_2	93	56—58	−2.9	0.82(A)	$C_{34}H_{41}N_5O_5SC_2F_3O_2$	59.7 (59.9)	5.6 (5.7)	9.3 (9.4)	4.2 (4.3)
(**35**)	$(Allyl)_2$-Tyr-D-Ala-Phe-Gly-ψ(CH_2S)-Phe-NH_2	87	92—94	−15.9	0.53(D)	$C_{38}H_{47}N_5O_5SC_2HF_3O_2$	59.8 (60.0)	6.2 (6.1)	8.8 (8.7)	4.1 (4.0)
(**36**)	$(Allyl)_2$-Tyr-D-Ala-Phe-Gly-ψ(CH_2S)-D-Phgly-NH_2	93	64—66	−1.8	0.52(D)	$C_{37}H_{45}N_5O_5SC_2HF_3O_2$	59.8 (59.6)	5.8 (5.8)	8.8 (8.9)	4.0 (4.1)

[a] Optical rotations: *c* 1.0 in methanol. [b] T.l.c. on silica gel: solvent systems (A—D).

study indicate that (i) the substitution of a more flexible methylenethio linkage for an amide bond results in the retention of biological activity; (ii) agonistic potencies of *N*-allyl derivatives of dermorphin analogues are low, indicating that their affinity to the opioid receptor is reduced by *N*-allyl substitution; (iii) the choice of opioid receptor subtype by *N*,*N*-diallyl derivatives may be different from that shown by their parent peptides; (iv) no relationship exists between the agonist activity of a given dermorphin analogue and the antagonist potency of its diallyl congener; and (v) surprisingly, in comparison with the inactive *N*,*N*-diallyl-Tyr-D-Ala-Phe-Gly-NH_2 (**27**), its dehydroxy analogue (**28**) is a moderately potent but highly selective δ-antagonist.

Experimental

H.p.l.c. analysis was performed on a Bruker liquid chromatograph LC21-C equipped with a Bruker LC313 UV variable-wavelength detector. Recording and quantification were accomplished using a chromatographic data processor (Epson computer FX-80X). A IB01 C-18 column (250 × 4.5 mm i.d. 5 μm particle size) was used in the h.p.l.c. system. All solvents used were u.v. spectroscopic grade and were filtered and degassed prior to use. Analytical determinations for deprotected peptides were carried out by a gradient made up of two solvents: A, 10% (v/v) acetonitrile in water; B, 60% (v/v) acetonitrile in water, both containing 4.5mM trifluoroacetic acid and 4.9mM triethylamine. The gradient programme used was as follows: linear gradient from 10% to 25% B in 10 min; isocratic 25% B for 5 min; linear gradient from 25% to 40% B in 8 min. Chromatography was performed at a flow rate of 1 ml min^{-1}: all analogues showed, by analytical h.p.l.c., less than 1% impurities while monitoring at 210 and 277 nm. The amino acid composition was determined with a Carlo Erba 3A29 amino acid analyser, after acid hydrolysis in constant-boiling HCl containing 1% phenol. T.l.c. was performed on precoated plates of silica gel F254 (from E. Merck) with use of the following solvent systems: butanol–acetic acid–water (6:1:5 v/v) (R_{FA}); ethyl acetate–pyridine–acetic acid–water (60:20:6:11) (R_{FB}); chloroform–methanol–benzene (17:2:1) (R_{FC}); chloroform–methanol (1:1) (R_{FD}); chloroform–methanol (9:1) (R_{FE}); acetone–chloroform (1:1) (R_{FF}). Spots were revealed by ninhydrin 1% (Merck), fluorescamine (Hoffman-La Roche), and/or chlorine reagent. Symbols and abbreviations used follow the IUPAC-IUB recommendations;[21] other abbreviations used are as follows: DCCI, dicyclohexylcarbodi-imide; HOBt, 1-hydroxybenzotriazole; DMF, dimethylformamide; OSu, *N*-hydroxysuccinimidyl ester; NMM, *N*-methylmorpholine; EtOAc, ethyl acetate; TFA, trifluoroacetic acid; THF, tetrahydrofuran.

All amino acid residues are L unless specifically stated otherwise.

Coupling Procedures. Method A.—To a stirred solution (0.5—0.8M) of Boc-protected amino acid or Boc-protected peptide (1 mmol) in DMF, 1 equiv. of NMM was added; the mixture was cooled to −10 °C, treated with isobutyl chloroformate (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—0.6M) 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 with brine, 0.5N $KHSO_4$, brine, 5% $NaHCO_3$, and brine successively. The organic phase was dried ($MgSO_4$), filtered, and evaporated to dryness. The residue was crystallized from appropriate solvents or purified by column chromatography.

Method B. To a solution of the carboxy component (2 mmol) in DMF (10 ml) was added the amino acid component (2 mmol), NMM (2 mmol if the amino component was in the protonated form), HOBt (2 equiv.), and DCCI (2.1 mmol) in the above order at 0 °C. The reaction mixture was stirred for 2 h at 0 °C and 24 h at room temperature; *N*,*N*-dicyclohexylurea was filtered off, and the solution was diluted with EtOAc (100 ml) and worked up as described in method A.

Deprotection. Method C. Boc and/or Bu^t 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 under reduced pressure at 0 °C, and the residue was triturated with ether or light petroleum; the resulting solid peptide was collected and dried.

Method D. Hydrogenation was 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.

N-*t-Butoxycarbonyl*-D-*alanylphenylalanylglycine* (**1**).—Boc-D-Ala-Phe-Gly-OBzl[9] (4.83 g, 10 mmol) in acetic acid (150 ml) was hydrogenated for 3 h (deprotection procedure D). The pure title compound was obtained by precipitation from methanol–ether (3.21 g, 81%), m.p. 138—140 °C, $[\alpha]_D^{21}$ −34.1° (*c* 1, in MeOH), R_{FB} 0.56 and R_{FD} 0.64 (Found: C, 57.9; H, 6.9; N, 10.8. $C_{19}H_{27}N_3O_6$ requires C, 58.0; H, 6.9; N, 10.7%).

N-*t-Butoxycarbonyl*-D-*alanylphenylalanylglycinamide* (**2**).—According to the general coupling procedure A, a mixed anhydride from Boc-D-Ala-Phe-Gly-OH (**1**) (6 mmol) was treated with a saturated THF solution of ammonia. The crude compound (2 g) was purified by silica gel column chromatography using 3% methanol in chloroform as the eluant, and recrystallized from ether (71%), m.p. 188—190 °C, $[\alpha]_D^{21}$ +19.6 (*c* 1, in MeOH), R_{FC} 0.42 and R_{FE} 0.78 (Found: C, 56.6; H, 9.6; N, 14.1. $C_{19}H_{38}N_4O_5$ requires C, 56.7; H, 9.5; N, 13.9%).

N-*t-Butoxycarbonyl*-N-*allyl*-O^4-*t-butyltyrosyl*-D-*alanylphenylalanylglycinamide* (**3**).—Boc-D-Ala-Phe-Gly-NH_2 (**2**) (1.9 g, 5 mmol) was treated with TFA (10 ml) for 30 min (procedure C). The resulting H-D-Ala-Phe-Gly-NH_2 trifluoroacetate (2.0 g, 5 mmol) in DMF (30 ml) was treated with *N*-t-butoxycarbonyl-*N*-allyl-O^4-t-butyltyrosine[22] (5 mmol) by the DCCI–HOBt method (coupling procedure B). The title compound was purified by silica gel column chromatography using 3% methanol in chloroform as the eluant: it was obtained as an oil (63%), $[\alpha]_D^{22}$ −43.0° (*c* 1, in MeOH), R_{FC} 0.60 and R_{FA} 0.83 (Found: C, 64.3; H, 7.7; N, 10.8. $C_{35}H_{49}N_5O_7$ requires C, 64.5; H, 7.6; N, 10.7%).

N,N-*Diallyl*-O^4-*t-butyltyrosyl*-D-*alanylphenylalanylglycinamide* (**4**).—This was prepared from *N*,*N*-diallyl-Tyr(Bu^t)-OH[23] and H-D-Ala-Phe-Gly-NH_2 trifluoroacetate (obtained by TFA treatment of the Boc derivative) according to coupling procedure B. The tetrapeptide was purified by silica gel column chromatography using chloroform and 10% methanol in chloroform as the eluants (85.5%), $[\alpha]_D^{21}$ −5.9° (*c* 1.5, in MeOH), R_{FA} 0.66, R_{FB} 0.83, and R_{FD} 0.79 (Found: C, 66.9; H, 7.6; N, 11.8. $C_{33}H_{45}N_5O_5$ requires C, 67.0; H, 7.7; N, 11.9%).

2-(2-*t-Butoxycarbonylamino*-3-*phenylpropylthio*)*acetic Acid* [Boc-Phe-ψ(CH_2S)-Gly-OH] (**5**).—2-t-Butoxycarbonylamino-3-phenylpropanol (Boc-phenylalaninol) (5 g, 20 mmol) was converted into the *O*-tosyl derivative by reaction with toluene-*p*-sulphonyl chloride (3.8 g, 20 mmol) in pyridine (20 ml) at 5 °C overnight. The solvent was evaporated off and the residue was dissolved in ethyl acetate and washed with saturated $NaHCO_3$,

water, 5% aqueous citric acid, and water. The ethyl acetate solution was dried (Na_2SO_4) and evaporated to dryness to leave an oil (7.1 g, 87%).

A solution of sodium mercaptoacetate (1.4 g, 10 mmol) in 1M NaOH (10 ml) was added dropwise to the above tosyl derivative (3.64 g, 9 mmol) in DMF (30 ml) and the reaction mixture was stirred overnight at 50—55 °C. The suspension was diluted with water (20 ml) and washed with ether (2 × 30 ml); the aqueous solution was acidified to pH 5 with solid citric acid and extracted with ethyl acetate (3 × 30 ml). The organic phase was washed with 5% aqueous citric acid and saturated aqueous NaCl, dried (Na_2SO_4), and evaporated to an oil (1.4 g, 48%), R_{FB} 0.59 and R_{FE} 0.35 (Found: C, 59.0; H, 7.2; N, 4.3. $C_{16}H_{23}NO_4S$ requires C, 59.1; H, 7.1; N, 4.3%). A sample of the above oil (325 mg, 1 mmol) was dissolved in ether (15 ml) containing dicyclohexylamine (0.2 ml, 1 mmol); precipitation of Boc-Phe-ψ(CH_2S)-Gly-OH dicyclohexylammonium salt occurred on standing at 0 °C (82.5%), m.p. 138—139 °C (lit.,[24] 140—141 °C).

(S)-2-*Bromo*-2-*phenylacetic Acid* (**6**).—Sodium nitrite (12 g, 0.14 mol) in water (25 ml) was added dropwise over 3 h to a stirred solution of L-phenylglycine (16.4 g, 0.125 mol) and potassium bromide (48.7 g, 0.41 mol) in 3M sulphuric acid (24.0 ml) at 0 °C. Stirring was continued for a further 60 min at 0 °C and overnight at room temperature. The product was extracted into ether (4 × 100 ml), back-washed with saturated aqueous sodium chloride and then dried. Evaporation of the ether left an oil which became a solid mass in the cold (20 g, 74%). A sample was crystallized from cyclohexane; m.p. 108—110 °C, $[\alpha]_D^{20}$ −4.5° (*c* 1.0, in MeOH) (Found: C, 44.4; H, 3.3; Br, 14.7. $C_8H_7O_2Br$ requires C, 44.7; H, 3.2; Br, 14.9%).

(S)-2-(2-*Aminoethylthio*)-3-*phenylpropionic Acid* [H-Gly-ψ(CH_2S)-Phe-OH] (**7**).—(*R*)-2-Bromo-3-phenylpropionic acid[25] (2.29 g, 10 mmol) was dissolved in 0.5M aqueous $NaHCO_3$ (60 ml, 30 mmol) and the solution purged with nitrogen. 2-Aminoethanethiol hydrochloride (2.27 g, 20 mmol) was added under nitrogen and the reaction solution was stirred for 24 h and acidified (pH 6) with ice-cold 6M HCl. A solid started to separate from the cold reaction solution; after 8 h this was filtered off, washed with cold water, ethanol, and then with ether. The solid was crystallized from ethanol (1.15 g, 51%), m.p. 197—199 °C, R_{FA} 0.55 and R_{FB} 0.50 (Found: C, 58.5; N, 6.6; N, 6.3; S, 14.3. $C_{11}H_{15}NO_2S$ requires C, 58.6; H, 6.7; N, 6.2; S, 14.2%).

(R)-2-(2-*Aminoethylthio*)-2-*phenylacetic Acid* [H-Gly-ψ(CH_2S)-D-Phgly-OH] (**8**).—This was prepared from (*S*)-2-bromo-2-phenylacetic acid and 2-aminoethanethiol as above. The pseudopeptide was crystallized from aqueous ethanol (56%), m.p. 163—165 °C, R_{FA} 0.51 and R_{FB} 0.46 (Found: C, 56.5; H, 6.1; N, 6.7. $C_{10}H_{13}NO_2S$ requires C, 56.8; H, 6.2; N, 6.6%).

N-*t*-*Butoxycarbonylglycyl*-ψ(CH_2S)-*phenylalaninamide* (**9**).—H-Gly-ψ(CH_2S)-Phe-OH (2.26 g, 10 mmol) was converted into the Boc derivative by reaction with $(Boc)_2O$ (3.27 g, 15 mmol) in butanol–water–1M NaOH (2:1:1 v/v) (40 ml) at room temperature for 3 h. The solvent was evaporated off, the residue was partitioned between ether and water, and the usual work-up was performed excluding the $NaHCO_3$ treatment. The resulting Boc-Gly-ψ(CH_2S)-Phe-OH (2.27 g, 7 mmol) was treated with a saturated THF solution of ammonia (procedure A). The title compound was crystallized from ether (43%), m.p. 129—131 °C, $[\alpha]_D^{20}$ −33.0° (*c* 1.0, in MeOH), R_{FC} 0.52 (Found: C, 59.0; H, 7.4; N, 8.7; S, 9.5. $C_{16}H_{24}N_2O_3S$ requires C, 59.2; H, 7.5; N, 8.6; S, 9.8%).

The other protected tetra- and penta-peptides were prepared in a similar manner to that described for the synthesis of (**3**) and (**4**). Physicochemical and analytical data referring to intermediate sequences and target peptides, outlined in Schemes 1—3, are summarized in Table 2.

Preparations of Free Peptides (**26**)—(**36**).—Each Boc-peptide analogue was deprotected according to procedure C. The resulting free peptide (1 mmol) was dissolved in 0.5M acetic acid (2 ml) and passed through a 2 × 40 cm Sephadex G-25 column, using system A as eluant. Evaporation of solvent from the appropriate combined fractions gave the peptide trifluoroacetates which were recrystallized from MeOH–Et_2O (80—85%). Characterizations of the final 11 analogues are summarized in Table 2.

Bioassays.—The bioassays based on inhibition of electrically evoked contractions of the GPI[17] and of the MVD[18] were carried out as reported in the literature.[10,13] A log dose-response curve was determined with dermorphin-(1-4)-NH_2 (**26a**) as standard for each ileum or vas preparation, and IC_{50} values (doses causing a depression of 50% of the electrically evoked contraction) of the opioid peptide analogues being tested were normalized according to a published procedure.[26] The effects of all analogues on the GPI and MVD were completely antagonized by naloxone.

The antagonistic effectiveness of new analogues and reference compound, H-Tyr-Gly-Gly-ψ(CH_2S)-Phe-Leu-OH, against either a μ- or δ-agonist was tested in either GPI or MVD preparations. The K_e values were determined against morphine and [D-Ala^2, D-Leu^5]enkephalin according to the usual method.[20] In these experiments the slopes of the Schild plots did not deviate significantly from unity.

Binding studies with guinea pig brain membrane preparations were carried out as described in detail elsewhere.[19] [^{3}H]DAGO, [^{3}H]DADLE, and [^{3}H]EKC at the concentration of 0.6nM were used as radioligands, and incubations were performed at 0 °C for 2 h. To determinate IC_{50} values (*i.e.*, values for 50% inhibition of specific [^{3}H]DAGO, [^{3}H]DADLE, and [^{3}H]EKC binding) of the peptide under examination, the compounds were added, in triplicate, to binding assays at at least six different concentrations. The IC_{50} values were calculated by probit analysis.

References

1 W. R. Martin, C. G. Eades, J. A. Thompson, R. E. Huppler, and P. E. Gilbert, *J. Pharmacol. Exp. Ther.*, 1976, **197**, 517.
2 P. C. Montecucchi, R. de Castiglione, and V. Erspamer, *Int. J. Pept. Protein Res.*, 1981, **17**, 316.
3 E. F. Hahn, J. Fishman, Y. Shiwaku, F. F. Foldes, H. Nagashima, and D. Duncalf, *Res. Commun. Chem. Pathol. Pharmacol.*, 1977, **18**, 1.
4 T. Oka, K. Negishi, M. Ueki, and T. Inazu, in 'Peptide Chemistry 1982,' ed. S. Sakakibara, Protein Research Foundation, Osaka, 1983, p. 177.
5 J. S. Shaw, L. Miller, M. J. Turnbull, J. J. Gormley, and J. S. Morley, *Life Sci.*, 1982, **31**, 1259.
6 P. Belton, R. Cotton, M. B. Giles, J. J. Gormley, L. Miller, J. S. Shaw, D. Timms, and A. Wilkinson, *Life Sci., Sup. I*, 1983, **33**, 443.
7 R. Cotton, M. B. Giles, L. Miller, J. S. Shaw, and T. Timms, *Eur. J. Pharmacol.*, 1984, **97**, 331.
8 R. Tomatis, S. Salvadori, and G. P. Sarto, in 'Peptides 1982,' eds. K. Blaha and P. Malons, W. de Gruyter, Berlin, 1983, p. 495.
9 S. Salvadori and R. Tomatis, *Eur. J. Med. Chem.*, 1983, **18**, 489.
10 S. Salvadori, G. Balboni, M. Marastoni, G. P. Sarto, and R. Tomatis, *Hoppe-Seyler's Z. Physiol. Chem.*, 1984, **365**, 1199.
11 S. Salvadori, M. Marastoni, G. Balboni, and R. Tomatis, *J. Med. Chem.*, 1986, **29**, 889.
12 M. Marastoni, S. Salvadori, G. Balboni, P. A. Borea, and R. Tomatis, *J. Med. Chem.*, 1987, **30**, 1538 and references cited therein.

13 S. Salvadori, G. P. Sarto, and R. Tomatis, *Arzneim. Forsch./Drug Res.*, 1984, **34**, 410.
14 J. A. Yankeelov, K. F. Folk, and D. J. Carothers, *J. Org. Chem.*, 1978, **43**, 1623.
15 N. Acton and A. Komoriya, *Org. Prep. Proced. Int.*, 1982, **14**, 381.
16 W. Konig and R. Geiger, *Chem. Ber.*, 1970, **103**, 788.
17 H. W. Kosterlitz and A. J. Watt, *Br. J. Pharmacol. Chemother.*, 1968, **33**, 226.
18 J. Hughes, H. W. Kosterlitz, and F. M. Leslie, *Br. J. Pharmacol.*, 1975, **53**, 371.
19 M. G. C. Gillan, H. W. Kosterlitz, and S. Paterson, *Br. J. Pharmacol.*, 1980, **70**, 481.
20 R. J. Tallarida, A. Cowan, and N. W. Adler, *Life Sci.*, 1979, **25**, 637.
21 IUPAC-IUB Joint Commission on Biochemical Nomenclature, *Eur. J. Biochem.*, 1984, **138**, 9.
22 R. T. Schuman, E. L. Smithwick, D. L. Smiley, G. S. Brooke, and P. D. Gesellchen, in 'Peptides: Structure and Function,' eds. V. J. Hruby and D. H. Rich, Pierce Chemical Company, Rockford, 1983, p. 143.
23 E.P. 0076557 A3. E.P. 82302471-6.
24 A. Spatola, personal communication.
25 M. T. Briggs and J. S. Morley, *J. Chem. Soc., Perkin Trans. 1*, 1979, 2138.
26 A. A. Waterfield, F. M. Leslie, J. A. H. Lord, N. Ling, and H. W. Kosterlitz, *Eur. J. Pharmacol.*, 1979, **58**, 11.

Received 12*th May* 1987; *Paper* 7/833