Articles

Evidence of the Preferential Involvement of μ Receptors in Analgesia Using Enkephalins Highly Selective for Peripheral μ or δ Receptors

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In order to study the preferential involvement of μ or δ receptors in the analgesic effects of enkephalins, several peptides which selectively interact with these two kinds of receptors in peripheral organs were synthesized. The inhibitory potency on the electrically stimulated mouse vas deferens (δ receptors) of the short peptide Tyr-D-Ala-Gly-NH-CH(CH_3)CH_2CH(CH_3)$_2$ (**6**) is 2100 times lower (IC_{50} = 1220 nM) than that of the longer and more hydrophilic peptide Tyr-D-Ser-Gly-Phe-Leu-Thr (**10**) (IC_{50} = 0.58 nM). In contrast, the IC_{50} values of all the synthesized compounds on the guinea pig ileum assay (μ receptors) are in the same range (100–360 nM). Likewise, their analgesic activities in mice, measured on the hot-plate test after intracerebroventricular injection, are similar. Therefore, the dissociation between antinociceptive properties in mice and potencies on the mouse vas deferens unambiguously reflects a preferential implication of μ receptors in analgesia. The possible involvement of brain δ receptors in behavioral effects is discussed.

The wide range of pharmacological effects elicited by opiates has been related to receptor heterogeneity.[1-5] So, taking into account the differences in pharmacological responses following structural modifications of the opiate pharmacophore, Martin et al.[6] have postulated the existence in the brain of μ, κ, and σ opiate receptors. This hypothesis was confirmed by demonstrating the presence in brain,[3,7-11] as well as in peripheral organs,[12,13] of at least two kinds of sites for radiolabeled analogues of the endogenous opiate-like peptides, enkephalins.[14] These compounds bind to a low-affinity site (K_D = 5–10 nM) where they are readily displaced by natural and synthetic opiates and to a high affinity site (K_D = 0.2–1 nM) which exhibits a high preference for peptide structures. From a comparison of the binding properties and the pharmacological potency on isolated organs, the low-affinity site has been related to the μ receptors or morphine receptors of the guinea pig ileum (GPI), and the high-affinity site has been related to the δ receptors or enkephalin receptors of the mouse vas deferens (MVD).[3,11,12]

In the series of natural and synthetic opiates, the analgesic activity is well-correlated to both the binding affinity in brain tissue and the potency on the electrically stimulated guinea pig ileum (GPI).[15,16] Therefore, the antinociceptive properties of these various compounds were related to their interaction with the brain μ receptors. In the case of peptides, such correlation does not appear as clearly.[5,17-19] Thus, the more active compounds Tyr-D-Ala-Gly-MePhe-Met-(O)^5-OH (FK 33-824)[20] and D-Met^2-Pro^5-enkephalinamide[21] strongly interact with the two kinds of sites, although they exhibit a preference for the μ receptors.[11,19] Therefore, in order to explore the possible involvement of δ receptors in analgesia, we have studied the antinociceptive potency of several peptides which exhibit similar activities on the GPI (μ receptors) but display an entirely opposite behavior on the MVD (δ receptors). These specific peptides have been recently synthetized following a rational approach.[22-24]

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Table I. Inhibitory Potencies (IC_{50}) of Morphine and Enkephalins on the Guinea Pig Ileum (GPI) and Mouse Vas Deferens (MVD) Assays

no.	compd	IC_{50} (GPI),[a] nM	IC_{50} (MVD),[a] nM	IC_{50} (GPI)/ IC_{50} (MVD)
1	morphine	70 ± 8 (6)	390 ± 30 (6)	0.18
2	Tyr-Gly-Gly-Phe-Met	200 ± 19 (8)	13 ± 1.50 (8)	15.00
3	Tyr-D-Ala-Gly-MePhe-Met(O)-OH	7.2 ± 1.3 (4)[b]	15.7 ± 3.77 (4)[b]	0.46
4	Tyr-D-Met-Gly-Phe-Pro-NH_2	21.9 ± 3.6 (4)[c]	23.1 ± 3.7 (4)[c]	0.95
5	Tyr-D-Ala-Gly-Phe-D-Leu	47.8 ± 4.4 (4)[b]	0.54 ± 0.09 (5)[b]	88.00
6	Tyr-D-Ala-Gly-NH-$CH(CH_3)CH_2CH(CH_3)_2$	182 ± 21 (5)	1220 ± 210 (6)	0.15
7	Tyr-D-Met-Gly-NH-$CH(CH_3)CH_2CH(CH_3)_2$	100 ± 12 (8)	1100 ± 135 (8)	0.09
8	Tyr-Gly-Gly-Phe-Met-Thr	380 ± 25 (4)	6.1 ± 2.0 (4)	62.00
9	Tyr-D-Ala-Gly-Phe-Met-Thr	240 ± 18 (5)	1.09 ± 0.5 (5)	220.00
10	Tyr-D-Ser-Gly-Phe-Leu-Thr	360 ± 28 (9)	0.58 ± 0.2 (8)	620.00

[a] The values are the means plus or minus SEM; the number of observations is given in parentheses. [b] From ref 11. [c] From ref 19.

Table II. Analgesic Activity (Hot-Plate Test) of Enkephalin Analogues after Intracerebroventricular Administration in Mice and Relative Potency to Morphine (= 1)

no.	compd	icv dose (mouse), nM	hind-paw lick latency time, s[a]	rel potency[b]
	controls		7.4 ± 1.1 (10)	
1	morphine	2.66	25.2 ± 6.3 (8)	1
3	Tyr-D-Ala-Gly-MePhe-Met(O)-OH	NT	NT	>100[f]
4	Tyr-D-Met-Gly-Phe-Pro-NH_2	NT	NT	25
5	Tyr-D-Ala-Gly-Phe-D-Leu	NT	NT	3[h]
6	Tyr-D-Ala-Gly-NH-$CH(CH_3)CH_2CH(CH_3)_2$[e]	1.98	25.0 ± 6.4 (8)	1-3
7	Tyr-D-Met-Gly-NH-$CH(CH_3)CH_2CH(CH_3)_2$[e]	1.77	16.8 ± 4.6 (10)	1-2
7	Tyr-D-Met-Gly-NH-$CH(CH_3)CH_2CH(CH_3)_2$[e]	5.31	30.7 ± 6.1 (10)	3-4
7	Tyr-D-Met-Gly-NH-$CH(CH_3)CH_2CH(CH_3)_2$[e]	5.31 + naloxone[c]	9.8 ± 4.0 (10)	
7	Tyr-D-Met-Gly-NH-$CH(CH_3)CH_2CH(CH_3)_2$[e]	5.31 + diprenorphine[d]	7.1 ± 0.9 (10)	
8	Tyr-Gly-Gly-Phe-Met-Thr	1.48	6.5 ± 2.1 (6)	>0.1
9	Tyr-D-Ala-Gly-Phe-Met-Thr	1.45	29.3 ± 6.2 (8)	2-4
10	Tyr-D-Ser-Gly-Phe-Leu-Thr	1.46	32.6 ± 5.2 (10)	2-4
10	Tyr-D-Ser-Gly-Phe-Leu-Thr	1.46 + naloxone[c]	10.5 ± 3.0 (10)	
10	Tyr-D-Ser-Gly-Phe-Leu-Thr	1.46 + diprenorphine[d]	6.6 ± 1.3 (10)	

[a] 15 min after icv injection of the various compounds in a constant volume of 5 μL of saline. The values are the means plus or minus SEM; the number of observations is given in parentheses. [b] The results for compounds 3-5 correspond to the ratio of AD_{50} values. For compounds 5-9, only a range of relative potencies can be given due to the absence of dose-response curves. [c] 1 mg/kg of naloxone was injected sc 15 min before icv treatment. [d] 0.5 mg/kg of diprenorphine was injected sc 15 min before icv treatment. [e] Corresponds to a mixture of *R* and *S* isomers at the level of the amide chain. [f] Reference 11. [g] Reference 40. [h] Reference 41.

Results and Discussion

The potency of opioid peptides and morphine on the electrically evoked contractions of the myenteric plexus longitudinal muscle preparation of guinea pig ileum and of mouse vas deferens is reported in Table I. As already discussed, the replacement of the phenylalanine moiety by an hydrophobic alkyl chain and the shortening of the sequence of enkephalins bearing a D-residue in position 2 lead to a large decrease in the potency of the MVD, whereas the affinity for the μ receptors of GPI remains similar to that of Met-enkephalin.[22] Therefore the decarboxylated peptides **6** and **7** can be considered as relatively specific ligands for the μ receptors. Contrarily, the lengthening of the sequence of enkephalins and the increase in their hydrophilic content lead to hexapeptides which show a high specificity for the δ opiate receptors of MVD.[23] So, Tyr-D-Ser-Gly-Phe-Leu-Thr (**10**) exhibits an inhibitory potency 620 times higher on MVD than on GPI. This compound is therefore much more specific than D-Ala^2-D-Leu^5-enkephalin (DADL; **5**), a peptide currently used as a typical δ agonist.[7,11,25] Furthermore, as already reported,[11,19] the peptides **3** and **4**, which display strong analgesic properties in vivo, interact preferentially with μ receptors, although they remain more active on δ receptors than morphine (Table I).

In order to circumvent a possible difference in pharmacokinetic properties of the studied peptides, the analgesic potency was checked after intracerebroventricular (icv) injection. On the other hand, since the purpose of this study was to compare the activity of various peptides as a function of their specificity for the two distinct receptors, complete dose–response curves were not absolutely required. Consequently, all the compounds were icv injected strictly under the same conditions (1 μg/5 μL per mouse), and their antinociceptive properties were determined using the hot-plate test.[26]

As shown in Table II, the potencies of the peptides **6**, **7**, **9**, and **10** are in the same range, whatever their specificity for the δ receptors. Thus, the compounds **6** and **10**, which are respectively 3 times less and 672 times more potent than morphine on the MVD assay, exhibit similar analgesic properties in mice.

The lack of activity of **8**, corresponding to the sequence 61–66 of β-endorphin, is in accordance with previous reports.[27] This feature is probably due to the breakdown of the Tyr–Gly bond by brain aminopeptidases,[28] since enkephalinase, the more specific degrading enzyme of

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endogenous enkephalins,[29,30] is inhibited by the addition of a Thr[6] residue.[23,31] So, despite the use of the icv route, the aminopeptidases action remains consequential. This could be due to the liberation of such enzymes from tissues damaged at the time of the injection or to a possible involvement of aminopeptidases in the metabolism of enkephalins. Furthermore, the slightly low potency of **7** as compared to **6** could be due to the transport mechanism, since this compound remains significantly active after 90 min as shown by the latency time on the jump test[26] (34.5 ± 4.5 s for **7**), whereas the analogue **6** displays a shorter action [14.5 ± 5.2 s (9.2 ± 4.0 for controls)].

The dissociation between antinociceptive properties of the studied peptides and their potencies on MVD assays indicates a preferential implication of the μ receptors in analgesia. This feature has been proposed by several authors,[8,10,11,32] but the results of the present study, especially the nearly equal analgesic effects of peptides **6** and **10** which present a very large difference in δ receptors affinity but a similar potency on μ receptors, unambiguously corroborates this hypothesis.

Moreover, a same concentration of either naloxone (a typical μ antagonist) or diprenorphine (a compound which exhibits only a threefold lower potency against enkephalins than against normorphine)[34] is required to obtain a complete reversal of the analgesic effect of **7** and **10** (Table II). Although these preliminary experiments were performed using unique and high doses of the two antagonists, they seem to indicate that the μ receptors are mainly involved for the in vivo effect of both **7** (a μ-specific derivative) and **10** (a highly preferential ligand for δ receptors). Indeed, if the analgesic responses in mice have required important δ-receptor stimulation, one should expect very different doses of naloxone and diprenorphine to antagonize the antinociceptive effects of **7** and **10**.[32,33] However, a definitive statement about the involvement of a unique population of receptors in analgesia should require a precise determination of the in vivo equivalents of pA_2 values[32] using several concentrations of the antagonists.

Nevertheless, all these findings could indicate that, in mice, the analgesic responses against the nociceptive stimuli induced hot-plate experiments does not require significant δ-receptor stimulation. Contrastingly, a number of studies show that the δ receptors may be involved in behavioral effects.[32,35-37,43,44] Although in brain the affinity of morphine for μ receptors is approximately 35 times higher than for the δ receptors,[11,12] it is very likely that at doses eliciting analgesia, morphine as well as synthetic opiates bind to the two kinds of receptors. Obviously, in humans, pain feeling as well as analgesia result from a complexity of events occuring in the central nervous system. Therefore, several secondary effects, such as euphoria, sedation, changes in locomotor activity, etc., could be mediated by the interaction of opiates with δ receptors. This assumption could be reinforced by the preponderance of δ receptors over μ receptors in the corpus striatum,[38] a region rich in dopamine receptors and, therefore, frequently implicated in mental illness. Accordingly, using Tyr-D-Ser-Gly-Phe-Leu-Thr (**10**), Chesselet et al. have recently shown[39] that presynaptic regulation of dopamine release in the caudate nucleus, a particular area of the striatum, seems to be mediated by δ receptors. Therefore, the synthesis of molecules which are thoroughly selective for one kind of site represents a very interesting and challenging problem.

Experimental Section

Biological Tests. Opioid activity was evaluated on GPI and on MVD as previously described.[42] Six different concentrations of each compound (six to eight assays for each) are tested for the inhibition of electrically induced contractions. IC_{50} values were determined by regression analysis. According to Kosterlitz,[11] methionine-enkephalin was used in each assay as the internal standard to avoid differences of sensitivity of each preparation. Analgesic activity was measured on the hot-plate test (65 ± 1 °C)[26] using swiss mice (20–22 g, Charles-Rivers) as previously described.[32,42] The compounds were freshly dissolved in saline and injected in a constant volume of 5 μL. The reaction times corresponding to the hind-paw lick were measured 15 min after treatment, and the reaction times corresponding to the adjusted jump[26] were measured after 90 min. If the control value exceeded 9 s, the animal was rejected. Naloxone (1 mg/kg), diprenorphine (0.5 mg/kg), or saline (0.2 mL) was given sc 15 min before icv treatment.

Chemistry. Methionine-enkephalin and protected amino acids are from Bachem (Switzerland). The peptides were prepared by the liquid-phase method using *tert*-butyloxycarbonyl (Boc) and methyl esters as protecting groups and dicyclohexylcarbodiimide (DCC) with hydroxybenzotriazole (HOBT) as coupling reagents.[42] The structure of the compounds and of all the intermediates were confirmed by ^1H NMR spectroscopy (Bruker WH 270 MHz). The purity was checked by thin-layer chromatography on (Merck) silica gel plates in the following solvent systems (v/v): A, chloroform-methanol (85:15); B, BuOH-AcOH-H_2O (4:1:1); C, chloroform-methanol (9:1); D, chloroform-methanol (7:3); E, 2-propanol-ammonia (7:3). Plates were revealed with iodine vapor or by spraying with buffered ninhydrin. At each step of the synthesis, the lack of significant racemization of a given peptide was checked by ^1N NMR spectroscopy at 270 MHz and by HPLC on a reverse-phase μBondapak C_{18} column (Waters) with CH_3CN/NH_4AcO buffer (pH 4.2) as solvent. The eluted peaks were monitored at 220 nm. Complete assignment of ^1H NMR signals of all the intermediary compounds was performed by classical double-resonance experiments.[45,46] Chemical shifts (in parts per

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million ± 0.02) relative to HMDS as internal reference were reported only for the final peptides **6**, **7**, **9**, and **10**. Melting points of the crystallized products are reported uncorrected. Analyses were given for the most relevant compounds (including intermediates), except for the trifluoroacetate salts which are too highly hygroscopic. In these cases, the purity of the obtained compounds was checked by TLC and HPLC, and the structure was unambiguously confirmed by ^1H NMR spectroscopy by the well-known methods already described for enkephalins.[45,46] The following abbreviations are used: THF, tetrahydrofuran; MeOH, methanol; $CHCl_3$, chloroform; EtOAc, ethyl acetate; DCC, dicyclohexylcarbodiimide; HOBT, 1-hydroxybenzotriazole; TFA, trifluoroacetic acid; Boc, *tert*-butyloxycarbonyl.

***N*-(*tert*-Butyloxycarbonyl)-L-tyrosyl-D-alanine Methyl Ester.** To a solution of *N*-(*tert*-butyloxycarbonyl)-L-tyrosine (1.69 g, 6 mmol) in anhydrous THF (15 mL), cooled at 0 °C, were added successively a solution of D-alanine methyl ester hydrochloride (0.84 g, 6 mmol) and triethylamine (0.85 mL) in $CHCl_3$ (15 mL), a solution of HOBT (0.92 g, 6 mmol) in THF (10 mL), a solution of DCC (1.24 g, 6 mmol) in $CHCl_3$ (5 mL). After 1 h, the mixture was allowed to come to room temperature and was stirred overnight. After removal of dicyclohexylurea and evaporation of solvents in vacuo, the residue was dissolved in EtOAc (25 mL) and washed successively with a saturated solution of NaCl (20 mL), a 10% solution of citric acid (3 × 20 mL), water (20 mL), a 10% solution of $NaHCO_3$ (3 × 20 mL) and, finally, with a saturated solution of NaCl (20 mL). The solvent was dried on Na_2SO_4 and evaporated in vacuo. This procedure is designated as standard treatment. The protected dipeptide was obtained as a white solid, recrystallized from EtOAc: yield 2.10 g (95%); TLC R_f (C) 0.45; mp 78–80 °C. Anal. Calcd for $C_{18}H_{26}N_2O_6$: C, 59.00; H, 7.15; N, 7.65. Found: C, 59.26; H, 7.28; N, 7.50.

***N*-(*tert*-Butyloxycarbonyl)-L-tyrosyl-D-alanine.** To a solution of the preceding compound (1.47 g, 4 mmol) in MeOH (10 mL) cooled at 0 °C, 8 mL of 1 N NaOH was added. The mixture was stirred at 0 °C for 1 h and at room temperature for 4 h. The solution was concentrated in vacuo, diluted with 10 mL of water, filtered, and acidified to pH 2 with 1 N HCl. After extraction of the aqueous solution by EtOAc, the organic layer was dried and evaporated in vacuo. This treatment is designated as "standard procedure for alkaline hydrolysis". The white solid was recrystallized from EtOAc, yielding 1.31 g (93%) of the pure protected dipeptide: mp 232–234 °C; TLC R_f (C) 0.91. Anal. Calcd for $C_{17}H_{24}N_2O_6$: C, 57.94; H, 6.87; N, 7.95. Found: C, 57.62; H, 6.68; N, 8.02.

***N*-(*tert*-Butyloxycarbonyl)-L-tyrosyl-D-alanylglycine Methyl Ester.** To a solution of *N*-(*tert*-butyloxycarbonyl)-L-tyrosine-D-alanine (1.06 g, 3 mmol) in anhydrous THF (10 mL), cooled in an ice–water bath, were added successively a mixture of glycine methyl ester hydrochloride (0.38 g, 3 mmol) and triethylamine (0.42 mL) in $CHCl_3$ (20 mL), a solution of HOBT (0.46 g, 3 mmol) in anhydrous THF (10 mL), and a solution of DCC (0.62 g, 3 mmol) in $CHCl_3$ (10 mL). After 1 h at 0 °C, the mixture was allowed to come to room temperature and stirred for 20 h. The reaction was worked up following the standard treatment and yielded a white solid, 0.90 g (70%); mp 159–160 °C; TLC R_f (C) 0.41. Anal. Calcd for $C_{20}H_{29}N_3O_7$: C, 56.72; H, 6.90; N, 9.92. Found: C, 56.96; H, 7.00; N, 9.76.

(*R*,*S*)-*N*-(*tert*-Butyloxycarbonyl)-L-tyrosyl-D-alanylglycine 1,3-Dimethylbutyl Amide. To a solution of the preceding compound (635 mg, 1.5 mmol) was added 10 mL of (*R*,*S*)-1,3-dimethylbutylamine. The mixture was allowed to stand at room temperature for a week. After removal of in vacuo of both the solvent and the excess of amine, the residue was triturated with ether until a white solid was obtained. This was collected, washed with ether, and dried. The product weighed 738 mg (98%); mp 100–102 °C; TLC R_f (C) 0.37. Anal. Calcd for $C_{25}N_{40}N_4O_6$: C, 60.95; H, 8.19; N, 11.38. Found: C, 61.10; H, 8.06; N, 11.20.

(*R*,*S*)-L-Tyrosyl-D-alanylglycine 1,3-Dimethylbutyl Amide Trifluoroacetate (6). The preceding compound (100 mg, 0.20 mmol) was dissolved in TFA (0.3 mL) at 0 °C. After 30 min, the mixture was allowed to come to room temperature and stirred for 30 min. The addition of ether (50 mL) led to the precipitation of crude compound **6**. The white solid was washed with ether (5 × 80 mL) and dried in vacuo: yield 86 mg (85%); TLC R_f (B) 0.80. NMR (Me_2SO-d_6) δ 3.90 (Tyr α H), 2.80 (β CH_2), 6.95 and 6.63 (Ar), 4.85 (Ala α H), 1.02 (CH_3), 3.57 (Gly CH_2); chain protons, δ 3.75 [CH(NH)], 1.23 and 1.09 (CH_2), 1.47 [CH(isopropyl)], 0.95 [CH_3(CH)], 0.78 [CH_3(isopropyl)]. HPLC (reversed phase μBondapak C_{18}), solvent CH_3CN/NH_4AcO, buffer pH 4.2, 40:60; flow rate 1.2 mL/min; retention time 252 s.

***N*-(*tert*-Butyloxycarbonyl)-D-methionylglycine Methyl Ester.** To a solution of *N*-(*tert*-butyloxycarbonyl)-D-methionine (1.25 g, 5 mmol) in anhydrous THF (30 mL), cooled in an ice–water bath, were added successively a mixture of glycine methyl ester hydrochloride (0.63 g, 5 mmol) and triethylamine (0.70 mL) in $CHCl_3$ (20 mL), a solution of HOBT (0.76 g, 5 mmol) in THF (10 mL), and a solution of DCC (1.03 g, 5 mmol) in $CHCl_3$ (10 mL). After the standard treatment, the protected dipeptide was obtained as a white powder: yield 1.13 g (70%); mp 90–91 °C; TLC R_f (A) 0.75. Anal. Calcd for $C_{13}H_{24}N_2O_5S$: C, 48.74; H, 7.55; N, 8.75; S, 9.99. Found: C, 48.95; H, 7.62; N, 8.62; S, 9.78.

D-Methionylglycine Methyl Ester Trifluoroacetate. The preceding compound was dissolved in TFA (4.5 mL) at 0 °C. After 30 min, the mixture was allowed to come to room temperature and stirred for 30 min. The addition of ether (50 mL) led to the precipitation of a white solid, which was washed with ether (5 × 80 mL) and dried in vacuo: yield 802 mg (85%); TLC R_f (B) 0.80.

***N*-(*tert*-Butyloxycarbonyl)-L-tyrosyl-D-methionylglycine Methyl Ester.** To a solution of *N*-(*tert*-butyloxycarbonyl)-L-tyrosine (620 mg, 2.2 mmol) in anhydrous THF (10 mL), cooled in an ice–water bath, were added successively a solution of the preceding compound (730 mg, 2.2 mmol) and triethylamine (0.31 mL) in $CHCl_3$ (20 mL), a solution of HOBT (0.34 g, 2.2 mmol) in THF (10 mL), and a solution of DCC (0.45 g, 2.2 mmol) in $CHCl_3$ (10 mL). After 1 h at 0 °C, the mixture was allowed to come to room temperature and then stirred overnight. The reaction was treated following the standard procedure, and a white solid was obtained: yield 530 mg (50%); mp 128–130 °C; TLC R_f (A) 0.70. Anal. Calcd for $C_{22}H_{33}N_3O_7S$: C, 54.65; H, 6.88; N, 8.69; S, 6.62. Found: C, 54.75; H, 7.02; N, 8.50; S, 6.92.

(*R*,*S*)-*N*-(*tert*-Butyloxycarbonyl)-L-tyrosyl-D-methionylglycine 1,3-Dimethylbutyl Amide. To a solution of the preceding compound (400 mg, 0.8 mmol) in methanol (5 mL), cooled in an ice–water bath, was added 5 mL of (*R*,*S*)-1,3-dimethylbutylamine. The ice bath was removed and the mixture was stirred for 8 days at room temperature. After evaporation of the solvent and of the excess of amine in vacuo, the residue was purified by gel filtration on LH20 with CH_3OH as solvent. A white solid, 410 mg (90%), was obtained: mp 95–96 °C; TLC R_f (A) 0.61. Anal. Calcd for $C_{27}H_{44}N_4O_6S$: C, 57.02; H, 7.80; N, 9.85; S, 6.33. Found: C, 56.86; H, 7.62; N, 10.05; S, 6.51.

(*R*,*S*)-L-Tyrosyl-D-methionylglycine 1,3-Dimethylbutyl Amide Trifluoroacetate (7). As for compound **6**, the *N*-(*tert*-butyloxycarbonyl) group was removed by TFA. From 221 mg (0.4 mmol) of the preceding compound, 190 mg of **7** (84%) was obtained as a white solid: TLC R_f (B) 0.55; NMR δ (Me_2SO-d_6) 3.95 (Tyr α H), 2.82 (β CH_2), 6.98 and 6.65 (Ar), 4.30 (Met α H), 1.68 (β CH_2), 2.15 (γ CH_2), 1.93 (SCH_3), 3.58 (Gly CH_2); chain protons, δ 3.75 [CH(NH)], 1.23 and 1.09 (CH_2), 1.47 [CH(isopropyl)], 0.95 [CH_3(CH)], 0.78 [CH_3(isopropyl)]. HPLC (reversed phase μBondapak C_{18}), solvent CH_3CN/NH_4AcO, buffer pH 4.2, 40:60; flow rate 1.2 mL/min; retention time 330 s.

***N*-(*tert*-Butyloxycarbonyl)glycyl-L-phenylalanine Methyl Ester.** To a solution of *N*-(*tert*-butyloxycarbonyl)glycine (5.25 g, 30 mmol) in anhydrous THF (30 mL), cooled in an ice–water bath, were successively added a mixture of L-phenylalanine methyl ester hydrochloride (6.47 g, 30 mmol) and triethylamine (4.2 mL) in $CHCl_3$ (40 mL), a solution of HOBT (4.60 g, 30 mmol) in THF (40 mL), and a solution of DCC (6.20 g, 30 mmol) in $CHCl_3$ (40 mL). After 1 h at 0 °C, the mixture was allowed to come to room temperature and stirred overnight. The reaction was treated following the standard procedure, and an oily residue, 10 g (99%), was obtained: single spot on TLC R_f (C) 0.45. Anal. Calcd for $C_{17}H_{24}N_2O_5$: C, 60.70; H, 7.19; N, 8.33. Found: C, 60.56; H, 7.26; N, 8.42.

***N*-(*tert*-Butyloxycarbonyl)glycyl-L-phenylalanine.** To a solution of the preceding compound (10 g, 30 mmol) in MeOH (60 mL) at 0 °C was added 30 mL of 1 N NaOH. The mixture was stirred at 0 °C for 45 min and at room temperature for 2.5

h. Then the reaction was treated following the standard procedure for alkaline hydrolysis. The product was recrystallized from EtOAc: yield 7.20 g (75%); mp 151–153 °C; TLC R_f (B) 0.90. Anal. Calcd for $C_{16}H_{22}N_2O_5$: C, 59.61; H, 6.88; N, 8.69. Found: C, 59.33; H, 6.95; N, 8.86.

***N*-(*tert*-Butyloxycarbonyl)glycyl-L-phenylalanyl-L-methionine Methyl Ester.** To a solution of *N*-(*tert*-butyloxycarbonyl)glycyl-L-phenylalanine (1.61 g, 5 mmol) in THF (30 mL), cooled in an ice–water bath, were added successively a solution of L-methionine methyl ester hydrochloride (1 g, 5 mmol) and triethylamine (0.7 mL) in $CHCl_3$ (20 mL) a solution of HOBT (0.74 g, 5 mmol) in THF (10 mL), and a solution of DCC (1.03 g, 5 mmol) in $CHCl_3$ (10 mL). After 1 h at 0 °C, the mixture was stirred at room temperature for 4 h. The reaction was treated as usual, and the obtained compound was recrystallized from EtOAc: yield 1.87 g (80%); mp 112–113 °C; TLC R_f (C) 0.62. Anal. Calcd for $C_{21}H_{33}N_3O_5S$: C, 57.39; H, 7.57; N, 9.56; S, 7.28. Found: C, 57.51; H, 7.62; N, 9.70; S, 7.21.

Glycyl-L-phenylalanyl-L-methionine Methyl Ester Trifluoroacetate. *N*-(*tert*-butyloxycarbonyl)glycyl-L-phenylalanyl-L-methionine methyl ester (1.87 g, 4 mmol) was dissolved in 6 mL of trifluoroacetic acid at 0 °C. After 20 min, the solution was allowed to come at room temperature and stirred for 30 min. Then the reaction was treated in the same manner as for **6**, yielding 1.75 g (90%) of a white powder: TLC R_f (D) 0.46.

***N*-(*tert*-Butyloxycarbonyl)-L-tyrosyl-D-alanylglycyl-L-phenylalanyl-L-methionine Methyl Ester.** To a solution of *N*-(*tert*-butyloxycarbonyl)-L-tyrosyl-D-alanine (1.06 g, 3 mmol) in anhydrous THF (20 mL), cooled at 0 °C, were successively added a solution of glycyl-L-phenylalanyl-L-methionine methyl ester trifluoroacetate (1.44 g, 3 mmol) and triethylamine (0.42 mL) in $CHCl_3$ (20 mL), a solution of HOBT (0.46 g, 3 mmol) in THF (10 mL), and a solution of DCC (0.62 g, 3 mmol) in $CHCl_3$ (10 mL). After 1 h at 0 °C, the mixture was stirred at room temperature for 24 h. The reaction was treated following the standard procedure, and the protected pentapeptide was recrystallized from EtOAc: yield 2 g (95%); mp 136–137 °C; TLC R_f (C) 0.70. Anal. Calcd for $C_{34}H_{47}N_5O_9S$: C, 58.19; H, 6.75; N, 9.98; S, 4.56. Found: C, 58.32; H, 6.86; N, 9.83; S, 4.50.

***N*-(*tert*-Butyloxycarbonyl)-L-tyrosyl-D-alanylglycyl-L-phenylalanyl-L-methionine.** To a solution of the preceding compound (1.7 g, 2.5 mmol) in MeOH (20 mL) was added 3.5 mL of 1 N NaOH at 0 °C. The mixture was stirred at 0 °C for 30 min and then at room temperature for 20 h. The reaction was treated following the standard procedure for alkaline hydrolysis, and recrystallization from EtOAc yielded the pure N-protected pentapeptide: yield 1.25 g (73%); mp 220–225 °C; TLC R_f (D) 0.51. Anal. Calcd for $C_{33}H_{45}N_5O_9S$: C, 57.63; H, 6.59; N, 10.18; S, 4.65. Found: C, 57.76; H, 6.75; N, 10.02; S, 4.80.

***N*-(*tert*-Butyloxycarbonyl)-L-tyrosyl-D-alanylglycyl-L-phenylalanyl-L-methionyl-*O*-*tert*-butyl-L-threonine Methyl Ester.** To a solution of *N*-(*tert*-butyloxycarbonyl)-L-tyrosyl-D-alanylglycyl-L-phenylalanyl-L-methionine (0.68 g, 1 mmol) in THF (10 mL), cooled in an ice–water bath, were added a solution of *O*-*tert*-butyl-L-threonine methyl ester hydrochloride (0.22 g, 1 mmol) and triethylamine (0.14 mL) in $CHCl_3$ (10 mL), a solution of HOBT (0.15 g, 1 mmol) in THF (5 mL), and a solution of DCC (0.20 g, 1 mmol) in $CHCl_3$ (5 mL). After 1 h at 0 °C, the mixture was allowed to come to room temperature and stirred for 48 h. The reaction was treated as usual, and a white solid, 0.80 g (93%), was obtained: TLC R_f (C) 0.35. Anal. Calcd for $C_{42}H_{62}N_6O_{11}S$: C, 58.72; H, 7.28; N, 9.78; S, 3.73. Found: C, 58.52; H, 7.42; N, 9.90; S, 3.66.

***N*-(*tert*-Butyloxycarbonyl)-L-tyrosyl-D-alanylglycyl-L-phenylalanyl-L-methionyl-*O*-*tert*-butyl-L-threonine.** To a solution of the preceding compound (0.8 g, 0.93 mmol) in MeOH (5 mL) was added 2 mL of 1 N NaOH at 0 °C. The mixture was stirred for 1 h at 0 °C and then at room temperature for 72 h. The reaction was treated following the standard procedure for alkaline hydrolysis and yielded the N-protected hexapeptide: yield, 0.47 g (60%); TLC R_f (B) 0.75. Anal. Calcd for $C_{41}H_{60}N_6O_{11}S$: C, 58.28; H, 7.16; N, 9.95; S, 3.79. Found: C, 58.16; H, 7.25; N, 9.92; S, 3.86.

L-Tyrosyl-D-alanylglycyl-L-phenylalanyl-L-methionyl-L-threonine (9). The preceding compound (0.42 g, 0.5 mmol) was dissolved at 0 °C in TFA (1.5 mL). The reaction mixture was stirred at 0 °C for 30 min and then at room temperature for 1 h. Ether (50 mL) was added to the well-stirred mixture. The precipitate was collected and washed with ether (6 × 50 mL). The crude product was purified by gel filtration on LH20 (Pharmacia) with MeOH as eluent. Fractions containing pure hexapeptide were evaporated in vacuo and lyophilized to yield 0.24 g (70%). The purity of the hexapeptide was checked by TLC R_f (B) 0.6. Anal. Calcd for $C_{32}H_{44}N_6O_9S$: C, 55.80; H, 6.44; N, 12.2; S, 4.65. Found: C, 55.68; H, 6.52; N, 12.32; S, 4.55. NMR (Me_2SO-d_6) δ 4.03 (Tyr α H), 2.93 and 2.75 (β H), 4.31 (Ala α H), 1.40 (CH_3), 3.57 (Gly α H), 4.43 (Phe α H), 2.93 and 2.75 (β H), 4.18 (Met α H), 1.83 (β H), 2.35 (γ H), 1.97 (SCH_3), 3.82 (Thr α H), 4.03 (β H), 0.98 (CH_3).

***N*-(*tert*-Butyloxycarbonyl)-L-tyrosyl-*O*-*tert*-butyl-D-serine Methyl Ester.** To a solution of *N*-(*tert*-butyloxycarbonyl)-tyrosine (3.37 g, 12 mmol) in THF (20 mL), cooled at 0 °C, were added successively a solution of *O*-*tert*-butyl-D-serine methyl ester hydrochloride (2.54 g, 12 mmol) and triethylamine (1.6 mL) in $CHCl_3$ (20 mL), a solution of HOBT (1.84 g, 12 mmol) in THF (90 mL), and a solution of DCC (2.47 g, 12 mmol) in $CHCl_3$ (10 mL). After 1 h at 0 °C, the mixture was allowed to come to room temperature and then stirred overnight. The reaction was treated as usual, and a white solid was obtained: yield 4.68 g (89%); mp 82–84 °C; TLC R_f (C) 0.55. Anal. Calcd for $C_{22}H_{34}N_2O_7$: C, 60.25; H, 7.82; N, 6.39. Found: C, 60.31; H, 7.90; N, 6.52.

***N*-(*tert*-Butyloxycarbonyl)-L-tyrosyl-*O*-*tert*-butyl-D-serine.** To a solution of *N*-(*tert*-butyloxycarbonyl)-L-tyrosyl-*O*-*tert*-butyl-D-serine methyl ester (2.19 g, 5 mmol) in MeOH (10 mL) was added 10 mL of 1 N NaOH at 0 °C. The mixture was stirred at 0 °C for 30 min and then at room temperature for 2 h. The reaction was treated following the standard procedure for alkaline hydrolysis and produced a white solid: yield 1.98 g (94%); mp 102–104 °C; TLC R_f (C) 0.52. Anal. Calcd for $C_{21}H_{32}N_2O_7$: C, 59.42; H, 7.60; N, 6.60. Found: C, 59.61; H, 7.71; N, 6.76.

***N*-(*tert*-Butyloxycarbonyl)glycyl-L-phenylalanyl-L-leucine Methyl Ester.** To a solution of *N*-(*tert*-butyloxycarbonyl)glycyl-L-phenylalanine (6.76 g, 21 mmol) in anhydrous THF (50 mL), cooled in an ice–water bath, were added successively a solution of L-leucine methyl ester hydrochloride (3.81 g, 21 mmol) and triethylamine (2.90 mL) in $CHCl_3$ (25 mL), a solution of HOBT (3.51 g, 21 mmol) in THF (30 mL), and a solution of DCC (4.33 g, 21 mmol) in $CHCl_3$ (25 mL). After 1 h at 0 °C, the mixture was allowed to come to room temperature and then stirred overnight. The reaction was treated as usual, and the protected tripeptide was recrystallized from EtOAc: yield 7.64 g (80%); mp 80–82 °C; TLC R_f (C) 0.70. Anal. Calcd for $C_{23}H_{35}N_3O_6$: C, 57.84; H, 7.39; N, 14.67. Found: C, 57.59; H, 7.45; N, 14.55.

Glycyl-L-phenylalanyl-L-leucine Methyl Ester Trifluoroacetate. A sample of the preceding compound (4.64 g, 10.3 mmol) was dissolved in 15 mL of trifluoroacetic acid at 0 °C. After 30 min, the solution was allowed to come to room temperature and then stirred for 30 min. Then the reaction was treated by the standard procedure to give 3.10 g (65%) of a white powder: TLC R_f (D) 0.5.

***N*-(*tert*-Butyloxycarbonyl)-L-tyrosyl-*O*-*tert*-butyl-D-serylglycyl-L-phenylalanyl-L-leucine Methyl Ester.** To a solution of *N*-(*tert*-butyloxycarbonyl)-L-tyrosyl-*O*-*tert*-butyl-D-serine (1.69 g, 4 mmol) in anhydrous THF (15 mL), cooled in an ice–water bath, were added successively a solution of glycyl-L-phenylalanyl-L-leucine methyl ester trifluoroacetate (1.85 g, 4 mmol) and triethylamine (0.56 mL) in $CHCl_3$ (15 mL), a solution of HOBT (0.61 g, 4 mmol) in THF (5 mL), and a solution of DCC (0.82 g, 4 mmol) in $CHCl_3$ (5 mL). After 1 h at 0 °C, the mixture was allowed to come to room temperature and then stirred overnight. The reaction was treated following the standard procedure and a white solid was obtained: yield 1.75 g (60%); mp 138–140 °C; TLC R_f (C) 0.65. Anal. Calcd for $C_{39}H_{57}N_5O_{10}$: C, 61.96; H, 7.60; N, 9.26. Found: C, 62.01; H, 7.65; N, 9.20.

***N*-(*tert*-Butyloxycarbonyl)-L-tyrosyl-*O*-*tert*-butyl-D-serylglycyl-L-phenylalanyl-L-leucine.** To a solution of the preceding compound (1.71 g, 2.27 mmol) in MeOH cooled in an ice–water bath was added 4.6 mL of 1 N NaOH. The mixture was stirred at 0 °C for 30 min and then at room temperature for 20 h. Then the reaction was treated following the standard

procedure for alkaline hydrolysis, and the product was obtained as a white solid: yield 1.34 g (80%); TLC R_f (D) 0.45. Anal. Calcd for $C_{38}H_{55}N_5O_{10}$: C, 61.52; H, 7.47; N, 9.44. Found: C, 61.36; H, 7.38; N, 9.51.

***N*-(*tert*-Butyloxycarbonyl)-L-tyrosyl-*O*-*tert*-butyl-D-serylglycyl-L-phenylalanyl-L-leucyl-*O*-*tert*-butyl-L-threonine Methyl Ester.** To a solution of *N*-(*tert*-butyloxycarbonyl)-L-tyrosyl-*O*-*tert*-butyl-D-serylglycyl-L-phenylalanyl-L-leucine (1.33 g, 1.8 mmol) in THF (15 mL), cooled in a ice-water bath, were added successively a solution of *O*-*tert*-butyl-L-threonine methyl ester hydrochloride (0.40 g, 1.8 mmol) and triethylamine (0.25 mL) in $CHCl_3$ (15 mL), a solution of HOBT (0.27 g, 1.8 mmol) in THF (10 mL), and a solution of DCC (0.37 g, 1.8 mmol) in $CHCl_3$ (10 mL). After 1 h at 0 °C, the mixture was allowed to come to room temperature and then stirred for 20 h. The reaction was treated following the standard procedure, and a white solid (1.52 g, 90%) was obtained, which showed a single spot on TLC R_f (C) 0.67; mp 158–160 °C. Anal. Calcd for $C_{47}H_{72}N_6O_{12}$: C, 61.82; H, 7.95; N, 9.20. Found: C, 61.65; H, 8.02; N, 9.33.

***N*-(*tert*-Butyloxycarbonyl)-L-tyrosyl-*O*-*tert*-butyl-D-serylglycyl-L-phenylalanyl-L-leucyl-*O*-*tert*-butyl-L-threonine.** To a solution of the preceding compound (1.51 g, 1.66 mmol) in MeOH cooled at 0 °C was added 4 mL of 1 N NaOH. The mixture was stirred at 0 °C for 30 min and then at room temperature for 36 h. Then an excess of 1 N NaOH (1.2 mL) was added and stirring continued for 24 h. The reaction mixture was treated following the standard procedure for alkaline hydrolysis and yielded the N,O-protected hexapeptide: yield 1.26 g (85%); mp 178–180 °C; TLC R_f (B) 0.8. Anal. Calcd for $C_{46}H_{70}N_6O_{12}$: C, 61.31; H, 8.05; N, 9.33. Found: C, 61.52; H, 8.11; N, 9.17.

Tyrosyl-D-serylglycyl-L-phenylalanyl-L-leucyl-L-threonine (10). *N*-(*tert*-butyloxycarbonyl)-L-tyrosyl-*O*-*tert*-butyl-D-serylglycyl-L-phenylalanyl-L-leucyl-*O*-*tert*-butyl-L-threonine (0.230 g, 0.29 mmol) was dissolved at 0 °C in TFA saturated with HCl (1 mL). The reaction mixture was stirred at 0 °C during 30 min and then at room temperature for 30 min. Ether (50 mL) was added to the well-stirred mixture. The precipitate was collected and washed with ether (5 × 50 mL). The crude product was purified by gel filtration on LH20 (Pharmacia) with MeOH as eluent. Fractions containing the pure hexapeptide were evaporated in vacuo and lyophilized to yield 0.18 g (90%). The purity of the hexapeptide was checked both by TLC [single spot (R_f (D) 0.49)], and by HPLC on a Waters apparatus [(reversed phase μBondapak C18); solvent CH_3CN/NH_4AcO, buffer pH 4.2, 20:80, flow rate 1.2 mL/min, retention time of the single peak 612 s]. Anal. Calcd for $C_{33}H_{46}N_6O_{10}$: C, 57.71; H, 6.75; N, 12.24. Found: C, 57.88; H, 6.91; N, 12.12. NMR (Me_2SO-d_6) δ 3.77 (Tyr α H), 2.86 and 2.64 (β H), 6.98 and 6.61 (Ar H), 4.14 (Ser α H), 3.52 and 3.45 (β H), 3.65 (Gly α H), 4.39 (Phe α H), 2.99 and 2.76 (β H), 7.19 (Ar H), 4.14 (Leu α H), 1.48 (β H and γ H), 0.79 (CH_3), 3.91 (Thr α H), 3.91 (β H), 0.89 (CH_3).

These chemical shifts correspond to the spectrum of **10** performed on the Zwitterionic form. The slightly different chemical shifts reported for **10** in a preliminary communication[23] correspond to the trifluoroacetate form.

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Nonquaternary Cholinesterase Reactivators. Dialkylaminoalkyl Thioesters of α-Ketothiohydroximic Acids as Reactivators of Diisopropyl Phosphorofluoridate Inhibited Acetylcholinesterase

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We have prepared a series of α-ketothiohydroximic acid thioesters and evaluated them in vitro with respect to their ability to reactivate (diisopropylphosphoryl)acetylcholinesterase. The compounds conform to the general formula $RC(=O)C(=NOH)S(CH_2)_nNR_2'\cdot HCl$, where R = CH_3, C_6H_5, 4-$CH_3OC_6H_4$, 4-$NO_2C_6H_4$; n = 2, 3; and R' = CH_3, C_2H_5, or i-C_3H_7. We also prepared 4-$BrC_6H_4C(=NOH)S(CH_2)_2N(C_2H_5)_2\cdot HCl$ and 4-$CH_3OC_6H_4C(=O)C(=NOH)S(CH_2)_2N(C_2H_5)_2\cdot CH_3I$ for comparison. The α-ketothiohydroximates exhibit oxime acid dissociation constants (pK_a) in the range 6.9 to 8.4, bracketing the value of pK_a = 7.9, believed to be optimal for acetylcholinesterase reactivation. The compounds are also good nucleophiles; bimolecular rate constants (k_n) for reaction with *p*-nitrophenyl acetate follow the expression $\log (k_n) = 6.7 - 0.69(14 - pK_a)$. The reactivation of (diisopropylphosphoryl)acetylcholinesterase is highly dependent on the α-ketothiohydroximate structure: 4-h incubation of inhibited enzyme at pH 7.6, 25 °C, with 1×10^{-3} M 4-$CH_3OC_6H_4C(=O)C(=NOH)S(CH_2)_3N(CH_3)_2\cdot HCl$ gives no detectable restoration of activity, whereas 4-$CH_3OC_6H_4C(=O)C(=NOH)S(CH_2)_2N(C_2H_5)_2\cdot HCl$ restores inhibited enzyme activity to 58% of control under identical conditions. With α-ketothiohydroximate in excess over inhibited enzyme, the kinetics of reactivation are governed by an equilibrium constant (K_r) for binding α-ketothiohydroximate to the inhibited enzyme and a nucleophilic displacement rate constant (k_r) for attack on phosphorus.

A variety of toxic organophosphorus (OP) esters owe their biological activity to phosphylation[1] of a serine hydroxyl at the active site of acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7), AChE.[2,3] Therapy for anti-AChE agent intoxication is based on coadministration of anticholinergics (e.g., atropine) to antagonize the effects of accumulated acetylcholine (ACh) and of AChE "reactivators" that displace the phosphyl residue from the active site and restore enzymatic activity.[4,5]

The last 30 years have witnessed considerable interest in elucidating the mechanism of AChE reactivation, and it is recognized[6,7] that the reaction proceeds as shown in

(1) We use the term "phosphylation" when we do not wish to distinguish between phosphorylation and phosphonylation.
(2) D. F. Heath, "Organophosphorus Poisons—Anticholinesterases and Related Compounds", Pergamon Press, New York, 1961.
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