$J$ = 9 Hz, 11 Hz, 55 Hz). Anal. ($C_{11}H_{10}FNO_3$) C, H.

**β-Lactamase Assay. Determination of Kinetic Constants.** The Michaelis–Menten constants $K_m$ and $V_{max}$ were determined by computerized microacidimetry. The experimental conditions were pH 7 and 37 °C. Penicillin G was used as a reference antibiotic, and values for the tested compounds were expressed relative to the hydrolysis of penicillin G.

**Determination of Values for $K_i$.** For the compounds that demonstrated little or no hydrolysis by the β-lactamase, we studied the inhibition constant $K_i$ by use of competition procedures. This study required an analysis of the rate of hydrolysis of a given substrate (e.g., penicillin G) in the presence of various concentrations of the compound, which thus behaved as an inhibitor. When inhibition of substrate hydrolysis was competitive and reversible, the presence of the inhibitor resulted in a reduced rate of substrate hydrolysis. This new rate remained essentially constant during a time when the concentration of substrate could be considered quite constant. The irreversible phase of inactivation was then studied as a function of time, i.e., the enzymatic activity was monitored after various periods of incubation of the enzyme with the inactivator at concentrations ranging from 1 to 100 μM.

**Registry No.** (±)-**2a**, 111773-40-1; (±)-*cis*-**2b**, 111773-41-2; (±)-*trans*-**2b**, 111773-43-4; (±)-**2c**, 111773-42-3; (±)-**3a**, 111773-35-4; **3b**, 111773-36-5; (±)-**3c**, 111773-37-6; **3d**, 111773-38-7; (±)-**3e**, 111773-39-8; **4**, 111773-24-1; (±)-**5a**, 111773-26-3; **5b**, 61444-65-3; (±)-**5** ($X^1 = X^2 = Br$, $X^3 = Cl$), 111773-27-4; **6**, 111773-25-2; (±)-**7a**, 111773-28-5; **7b**, 111793-83-0; (±)-**7c**, 111773-29-6; **7d**, 111773-31-0; **8**, 111773-30-9; (±)-**9a**, 111773-32-1; **9b**, 111773-33-2; (±)-**9c**, 111793-84-1; **9d**, 111793-85-2; (±)-**9e**, 111793-86-3; (±)-**10**, 111773-34-3; $BrCH_2COCO_2Et$, 70-23-5; β-lactamase, 9073-60-3.

# Investigation of the Structural Parameters Involved in the μ and δ Opioid Receptor Discrimination of Linear Enkephalin-Related Peptides[1]

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The previous rules proposed for selective recognition of μ and δ opioid receptors by modified enkephalins were investigated through an extensive structure–activity study. Thus, modifications of the sequence of TRIMU 4 (Tyr-D-Ala-Gly-NHCH($CH_3$)$CH_2$CH($CH_3$)$_2$, a peptide that exhibits μ selectivity close to that of DAGO (Tyr-D-Ala-Gly-N(Me)Phe-Gly-ol), were performed for two positions, 2 and 4, critical for μ recognition. The drastic loss of potency following introduction of L-Ala or Aib in position 2 emphasizes the importance of the stereochemistry and the steric size of the $X^2$ amino acid for optimal μ binding. The enhancement of the intrinsic flexibility of the C-terminal alkyl chain of TRIMU 4 through removal of a methyl group leads to TRIMU 5 (Tyr-D-Ala-Gly-NHCH$_2$CH$_2$CH($CH_3$)$_2$), a peptide with a μ selectivity similar to that of DAGO. In contrast, introduction of an *O-tert*-butyl $Ser^2$ residue increases affinity for δ receptors. In the hexapeptide series derived from DSLET (Tyr-D-Ser-Gly-Phe-Leu-Thr), a D-$Thr^2$ moiety was shown to be very efficient in improving δ recognition and δ selectivity appeared also to be modulated by the nature of the sixth residue. The potencies of the 24 peptides studied to inhibit the electrically evoked contractions of the GPI or MVD are relatively well correlated with their affinities for brain μ or δ receptors labeled with [$^3$H]DAGO or [$^3$H]DSLET, respectively. Moreover, the analgesic potency (hot plate test) of the peptides is related to their affinity for rat brain μ receptors. The wide range of receptor affinities exhibited by the compounds reported here could be useful to study the physiological role of μ and δ receptors.

The multiplicity of pharmacological effects following opioid administration (analgesia, respiratory modification, euphoria, addiction) could be related to the nondiscriminate interaction of these compounds with several types of receptors.[2] This assumption is supported by the existence of different binding sites for morphine and the endogenous morphine-like peptides.[3] Indeed, it is now well established that [Met]- and [Leu]enkephalin interact in the central nervous system, as well as in peripheral organs, with at least two types of receptors designated μ and δ.[3] Among various physiological functions, the μ type seems to be preferentially involved in pain control at the supraspinal level[4-8] while the δ type may be especially implicated in the behavioral effects of opioids.[9-12] Nevertheless, unambiguous elucidation of the physiological role of each receptor type requires ideally the use of completely selective effectors (agonists and antagonists).

The dual interaction of enkephalins with two distinct binding sites means that, as classical neurotransmitters, the endogenous opioid peptides are able to adopt different conformations at different receptor sites.[13] This is con-

(1) Symbols and abbreviations are in accordance with recommendations of the IUPAC–IUB Joint Commission on Biochemical Nomenclature: *Biochem. J.* **1984**, *219*, 345. The following other abbreviations have been used: FAB, fast atom bombardment; THF, tetrahydrofuran; MeOH, methanol; $CHCl_3$, chloroform; EtOAc, ethyl acetate; $Et_2O$, ether; DCC, cyclohexylcarbodiimide; HOBT, 1-hydroxybenzotriazole; TFA, trifluoroacetic acid; Boc, *tert*-butyloxycarbonyl; Z, benzyloxycarbonyl; Aib, 2-aminoisobutyric acid; Pen, penicillamine; Az-Gly, $NH_2NHCOOH$; [Met]enkephalin, Tyr-Gly-Gly-Phe-Met; [Leu]enkephalin, Tyr-Gly-Gly-Phe-Leu; Metkephamid, Tyr-D-Ala-Gly-Phe-N(Me)-Met-$NH_2$.

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**Table I.** Inhibitory Potencies ($IC_{50}$) of Morphine and Opioid Peptides on the Guinea Pig Ileum (GPI) and Mouse Vas Deferens (MVD)[a]

| compounds | $IC_{50}$, nM | | |
|---|---|---|---|
| | GPI | MVD | $IC_{50}$ (MVD)/$IC_{50}$ (GPI) |
| 1, morphine | 70 ± 8 | 309 ± 30 | 5.55 |
| 2, [Met]enkephalin | 200 ± 19 | 13.0 ± 1.5 | 0.065 |
| 3, Tyr-D-Ala-Gly-NHCH($CH_3$)$CH_2$CH($CH_3$)$_2$ (TRIMU 4) | 237 ± 56 | 1050 ± 90 | 4.35 |
| 4, Tyr-Aib-Gly-NH-CH-($CH_3$)-$CH_2$-CH($CH_3$)$_2$ | 1850 ± 120 | 4720 ± 530 | 2.56 |
| 5, Tyr-L-Ala-Gly-NH-CH($CH_3$)-$CH_2$-CH($CH_3$)$_2$ | ≥50000 | ≥50000 | |
| 6, Tyr-D-Ser-Gly-NH-CH($CH_3$)-$CH_2$-CH($CH_3$)$_2$ | 718 ± 48 | 990 ± 143 | 1.37 |
| 7, Tyr-D-Ser(*O-t*-Bu)-Gly-NH-CH($CH_3$)-$CH_2$-CH($CH_3$)$_2$ | 111 ± 29 | 105 ± 25 | 0.94 |
| 8, Tyr-D-Ala-Gly-N(Et)-CH($CH_3$)-$CH_2$-CH($CH_3$)$_2$ | 216 ± 15 | 1800 ± 40 | 8.33 |
| 9, Tyr-D-Ala-Gly-NH-$CH_2$-$CH_2$-CH($CH_3$)$_2$ (TRIMU 5) | 323 ± 62 | 3410 ± 310 | 10.53 |
| 10, Tyr-D-Met-Gly-NH-$CH_2$-$CH_2$-CH($CH_3$)$_2$ | 154 ± 9 | 733 ± 61 | 4.76 |
| 11, Tyr-D-Ala-Gly-N(Me)-Phe-Gly-01 (DAGO) | 11.5 ± 0.4 | 76.1 ± 6.7 | 6.66 |
| 12, Tyr-D-Ala-Gly-N(Me)-Leu-Gly-01 | 379 ± 62 | 3180 ± 150 | 8.33 |
| 13, Tyr-D-Ser-Gly-Phe-Leu-Thr (DSLET) | 406 ± 46 | 0.40 ± 0.04 | 0.001 |
| 14, Tyr-Azgly-Gly-Phe-Leu-Thr | 14000 ± 500 | 38.0 ± 3.3 | 0.003 |
| 15, Tyr-D-Thr-Gly-Phe-Leu-Thr (DTLET) | 460 ± 60 | 0.150 ± 0.012 | 0.0003 |
| 16, Tyr-β-OH-Nva-Gly-Phe-Leu-Thr | 21.3 ± 5.7 | 0.05 ± 0.01 | 0.002 |
| 17, Tyr-D-Ser-Gly-Phe-Leu-Leu (DSLEL) | 257 ± 28 | 2.45 ± 0.80 | 0.009 |
| 18, Tyr-D-Thr-Gly-Phe-Leu-Leu | 260 ± 38 | 0.410 ± 0.037 | 0.0015 |
| 19, Tyr-D-Ser-Gly-Phe-Leu-des-COOH-Thr | 64 ± 12 | 3.45 ± 0.13 | 0.054 |
| 20, Tyr-D-Thr-Gly-Phe-Leu-Ser | 99 ± 12 | 0.25 ± 0.04 | 0.0025 |
| 21, Tyr-D-Thr-Gly-Phe-Leu-Hyp (DSLEH) | 88 ± 10 | 1.10 ± 0.07 | 0.012 |
| 22, Tyr-D-Thr-N(Me)-Phe-Leu-Thr | 93.2 ± 15 | 7.30 ± 0.30 | 0.078 |
| 23, Tyr-D-Thr-Gly-Phe-N(Me)-Leu-Thr | 224 ± 21 | 1.33 ± 0.09 | 0.006 |
| 24, Tyr-D-Pen-Gly-Phe-Pen | 10000 ± 400 | 2.50 ± 0.50 | 0.00025 |

[a] The values are the means ± SEM of four to eight independent determinations.

sistent with the high flexibility of these short pentapeptides, which contain two successive glycine residues permitting many low-energy conformational changes that may be required to fit the μ or the δ receptors through a zipper mechanism.[14-16] A classical way to obtain selective compounds has been to modify the sequence of the native peptide by introduction of residues that enhance the interaction with a single type of receptor. Following this strategy, we designed TRIMU 4, Tyr-D-Ala-Gly-NHCH($CH_3$)$CH_2$CH($CH_3$)$_2$,[17] a highly selective μ ligand and two linear hexapeptides DSLET, Tyr-D-Ser-Gly-Phe-Leu-Thr,[18] and DTLET, Tyr-D-Thr-Gly-Phe-Leu-Thr,[19] which behave as δ probes. Another approach to obtain highly selective opioid peptides has been centered on the restriction of conformation through peptide cyclization (review in ref 20 and 21). In agreement with our proposed structural requirements for specific interaction with μ and δ receptors,[13] most of the cyclic enkephalins reported so far have exhibited a rather high μ selectivity.[20] The exception is represented by two cyclic pentapeptides, Tyr-D-Pen-Gly-Phe-D-Pen and Tyr-D-Pen-Gly-Phe-L-Pen, which appear more δ selective than the linear hexapeptides DSLET and DTLET,[22,23] but have been shown to exhibit a weaker affinity for the δ receptors of rat brain.[24-27]

Consequently, it was of great interest to investigate in more detail our previously reported series of μ[5,17] and δ linear compounds.[19] Furthermore, to study the pharmacological properties of one receptor type, it is useful to possess several series of compounds belonging to different chemical classes and exhibiting various degrees of selectivities to ensure that the observed preferences are not related only to pharmacokinetic differences. In this paper, we report the modulation of the cross-reactivity for μ and δ opioid receptors of peptides derived from TRIMU 4 (μ ligand) and DSLET (δ ligand). The pharmacological profile of the compounds was determined from: (i) inhibition of the electrically evoked contractions of the guinea pig ileum (GPI) and the mouse vas deferens (MVD), used as relatively selective bioassays for μ and δ receptors, respectively, and (ii) inhibition of binding in rat brain tissue of [$^3$H]DAGO as a specific μ ligand[28] and [$^3$H]DSLET as a δ marker.[29]

The selectivity of compounds such as DSLET and DTLET was confirmed by their ability to preserve almost exclusively δ receptors of the MVD from inactivation by the nonspecific alkylating agent β-chlornaltrexamine.[30]


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Table II. Abilities of Morphine and Opioid Peptides To Inhibit the Binding of [$^3$H]DAGO and [$^3$H]DSLET from Rat Brain Tissue

| compound | $K_i$,[a] nM | | $K_i$ ([$^3$H]DSLET)/$K_i$ [($^3$H]DAGO) |
|---|---|---|---|
| | [$^3$H]DAGO | [$^3$H]DSLET | |
| 1, morphine | 2.15 ± 0.36 | 280 ± 43 | 130 |
| 2, [Met]enkephalin | 27.5 ± 8.0 | 7.30 ± 2.10 | 0.27 |
| 3, Tyr-D-Ala-Gly-NH-CH-$(CH_3)$-$CH_2$-$CH(CH_3)_2$ (TRIMU 4) | 17.0 ± 3.2 | 480 ± 96 | 28 |
| 4, Tyr-Aib-Gly-NH-CH-$(CH_3)$-$CH_2$-$CH(CH_3)_2$ | 171 ± 37 | 6880 ± 440 | 40 |
| 5, Tyr-L-Ala-Gly-NH-$CH(CH_3)$-$CH_2$-$CH(CH_3)_2$ | NT | NT | |
| 6, Tyr-D-Ser-Gly-NH-$CH(CH_3)$-$CH_2$-$CH(CH_3)_2$ | 43.0 ± 4.6 | 1370 ± 270 | 40 |
| 7, Tyr-D-Ser(*O*-*t*-Bu)-Gly-NH-$CH(CH_3)$-$CH_2$-$CH(CH_3)_2$ | 13.7 ± 1.3 | 39 ± 12 | 2.8 |
| 8, Tyr-D-Ala-Gly-N(Et)-$CH(CH_3)$-$CH_2$-$CH(CH_3)_2$ | 35.5 ± 6.3 | 3450 ± 190 | 97 |
| 9, Tyr-D-Ala-Gly-NH-$CH_2$-$CH_2$-$CH(CH_3)_2$ (TRIMU 5) | 10.0 ± 2.1 | 1020 ± 160 | 109 |
| 10, Tyr-D-Met-Gly-NH-$CH_2$-$CH_2$-$CH(CH_3)_2$ | 15.0 ± 3.0 | 600 ± 125 | 40 |
| 11, Tyr-D-Ala-Gly-N(Me) Phe-Gly-01 (DAGO) | 3.9 ± 0.8 | 700 ± 95 | 179 |
| 12, Tyr-D-Ala-Gly-N(Me)Leu-Gly-01 | 116 ± 23 | 3810 ± 690 | 33 |
| 13, Tyr-D-Ser-Gly-Phe-Leu-Thr (DSLET) | 31.0 ± 5.0 | 4.80 ± 0.80 | 0.15 |
| 14, Tyr-Azgly-Gly-Phe-Leu-Thr | 9790 ± 50 | 395 ± 72 | 0.040 |
| 15, Tyr-D-Thr-Gly-Phe-Leu-Thr (DTLET) | 25.3 ± 2.5 | 1.35 ± 0.15 | 0.053 |
| 16, Tyr-β-OH-Nva-Gly-Phe-Leu-Thr | 16.0 ± 2.5 | 2.2 ± 0.9 | 0.14 |
| 17, Tyr-D-Ser-Gly-Phe-Leu-Leu (DSLEL) | 158 ± 9 | 9.12 ± 1.61 | 0.058 |
| 18, Tyr-D-Thr-Gly-Phe-Leu-Leu | 38.0 ± 4.0 | 2.65 ± 0.36 | 0.070 |
| 19, Tyr-D-Ser-Gly-Phe-Leu-des-COOH-Thr | 9.20 ± 1.80 | 5.60 ± 0.70 | 0.61 |
| 20, Thr-D-Thr-Gly-Phe-Leu-Ser | 41.9 ± 6.8 | 4.18 ± 0.54 | 0.10 |
| 21, Tyr-D-Thr-Gly-Phe-Leu-Hyp (DSLEH) | 43.8 ± 8.3 | 7.60 ± 0.39 | 0.17 |
| 22, Tyr-D-Thr-Gly-N(Me)-Phe-Leu-Thr | 61.3 ± 11.1 | 30.0 ± 7.6 | 0.49 |
| 23, Tyr-D-Thr-Gly-Phe-N(Me)-Leu-Thr | 157 ± 11 | 30.7 ± 3.2 | 0.20 |
| 24, Tyr-D-Pen-Gly-Phe-Pen | 873 ± 210 | 10.9 ± 1.2 | 0.012 |

[a] The $K_i$ values are the means ± SEM for three to six independent determinations in triplicate. Each $K_i$ value was obtained from computer analysis of Hill plots with nine concentrations of unlabeled ligand.

Finally, the pharmacological interest of these compounds was tested by evaluation of their analgesic potency on the hot plate test after icv administration in mice.

## Results and Discussion

The potency of the synthesized opioid peptides on the electrically evoked contractions of the myenteric plexus longitudinal muscle preparation of guinea pig ileum and mouse vas deferens is reported in Table I. The activity at the central level was evaluated by the ability of the various peptides to inhibit the binding of [$^3$H]DAGO and [$^3$H]DSLET to rat brain membranes (Table II).

**Structure–Activity Relationships (SAR) for μ Receptor Recognition.** As previously discussed, TRIMU 4,[17] a short peptide that exhibits a μ selectivity close to that of DAGO,[28] was selected as a starting compound for SAR investigation of μ-receptor recognition. This peptide is devoid of the Phe ring, which occurs in residue 4 of the enkephalins and as the third residue in β-casomorphin and dermorphin. It can be noticed that most of the classical opiates do not contain a second aromatic ring (ethylketazocine, etorphine, levorphanol, naloxone).

In the present study, modifications of the sequence of TRIMU 4 were performed at two critical positions (2 and 4) for μ-site recognition.[13] Therefore, the peptides synthesized are characterized by a Tyr-X sequence followed by a glycine residue bearing a C-terminal short alkyl chain. These structural characteristics are assumed to give many potential conformations to these compounds. Compounds **4–8** prepared with (*R*,*S*)-1,3-dimethylbutylamine were obtained as diastereoisomeric peptides, which were not separated for biological studies.

The influence of the nature of the side chain of the second residue was first explored. The drastic loss of potency to both opioid receptors following the replacement of D-Ala in **3** by Aib in **4** and especially by L-Ala in **5** shows that with the two latter amino acids, the energetically accessible regions of the φ, ψ space are not compatible with the biologically active conformations reached by **3** in μ or δ binding sites. Similar results were observed by Shaw et al. with [Leu]enkephalin analogues.[31]

The occurrence in μ receptors of the previously proposed lipophilic locus for the side chain of $X^2$ residues[13] is supported by the pharmacological profile of compounds **6**, **7**, and **10**. Thus, in the former, the hydrophilic D-$Ser^2$ residue caused a 3-fold decrease in potency in the GPI assay. However, the introduction of a *tert*-butyl group on the $Ser^2$ hydroxyl group in **7** also induced an increase in the affinity for δ receptors (Tables I and II). This effect could be due to a modification of the peptide backbone conformation improving the recognition of the δ site through the zipper mechanism.[13-15] This assumption seems to be supported by the significant differences occurring in the NMR spectra of **6** and **7** monitored in strictly identical conditions. Thus, as compared to **6**, the introduction of a *tert*-butyl group in **7** induced a 0.1 ppm downfield shift of the α-Tyr proton, contrasting with the 0.07 ppm upfield shift undergone by the NH of Gly. Moreover, the β-protons of Tyr and D-Ser residues, which occurred under two well-separated peaks in the spectrum of **6**, were found under the form of degenerated signals in **7**. Finally, the occurrence of differences in the averaged conformations of **6** and **7** is supported by the $^3J_{NH-H_\alpha}$ values of the seryl residues, which were $J$ = 5.4 Hz and $J$ = 8.1 Hz in **6** and **7**, respectively. Moreover, the conformational differences were confirmed by the NOE peaks observed in the ROESY experiments.[32]

The second structural requirement, previously shown to be essential for opioid receptors differentiation, is the nature of the fourth residue.[13] The aromatic side chain of $Phe^4$ is very important for δ vs μ activity, as shown by the shift toward μ selectivity following its replacement by an aliphatic hydrophobic residue.[17] The importance of the fourth residue is corroborated by the biological results obtained for compounds **3** and **9**. Indeed, the removal of a methyl group and of the associated asymmetrical carbon

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in the C-terminal chain of TRIMU 4 caused only slight changes in the GPI assay but a 3-fold loss of potency in the MVD test. This led to an interesting compound named TRIMU 5, which displays about 10 times and 100 times higher potency for the peripheral and central μ receptors, respectively, than for the corresponding δ receptors. As compared to DAGO, TRIMU 5 displays a 2-fold increase in μ selectivity at the peripheral level but DAGO remains slightly more selective for the μ receptors of rat brain tissue (Table II). This apparent discrepancy could be related to differences in intrinsic activity of peptides belonging to different chemical classes,[33] emphasizing the necessity for various probes to explore the physiological relevance of one type of receptor.

Ethylation of the amide group of the Phe$^4$ residue in modified enkephalins was shown to induce a large increase in δ affinity.[34] In the present series, introduction of this modification in compound **8** did not change drastically the pharmacological profile. Surprisingly, the substitution of a leucine residue for the phenylalanine residue in **12** did not produce the expected enhancement in μ selectivity but led to a large loss of potency on all tests.

**Structure–Activity Relationships for δ Receptor Recognition.** DSLET, the first described opioid peptide exhibiting a significant δ selectivity,[18] was selected as the parent compound for the structure–activity study. Among the structural requirements previously shown to enhance δ selectivity,[5,18] the occurrence of a D-Ser$^2$ residue in DSLET was critical, probably through adverse interaction of the hydroxylated side chain with the corresponding hydrophobic subsite of the μ receptor.[13]

Taking these results in account, the D-Ser$^2$ residue of DSLET was first replaced by AzGly, another relatively hydrophilic residue (compound **14**). This led to an increase in δ selectivity, unfortunately associated with a loss of affinity for both kinds of sites, in accordance with previous reports in the series of [Leu]enkephalin analogues.[31] The enhancement in the size of the hydroxylated chain caused by the introduction in DTLET (**15**) of a D-Thr$^2$ residue for the D-Ser$^2$ residue produced a 3-fold enhancement of δ selectivity. As already reported,[24] DTLET behaves as the most efficient δ agonist reported so far and remains, therefore, extensively used in spite of its nonperfect specificity. These results corroborate those obtained with the Metkephamid series[35] in which the D-Thr$^2$ analogue was shown to be more potent in the MVD assay than compounds with D-Ser$^2$ or D-Ala$^2$ residues. Moreover, with the aim of increasing further the δ-inducing effect of Thr$^2$, the side chain of this residue was extended by an additional methylene group in compound **16**. This introduction of a β-hydroxynorvaline in position 2 provided, as expected, a highly potent δ agonist in the MVD assay, but this effect was associated with an increase in μ potency, likely related to the hydrophobic interaction between the aliphatic part of the chain and the μ pharmacophore. In fact, it seems that for δ receptor recognition, a Thr$^2$ residue represents a good compromise in terms of the hydrophilic–hydrophobic balance of the peptide.

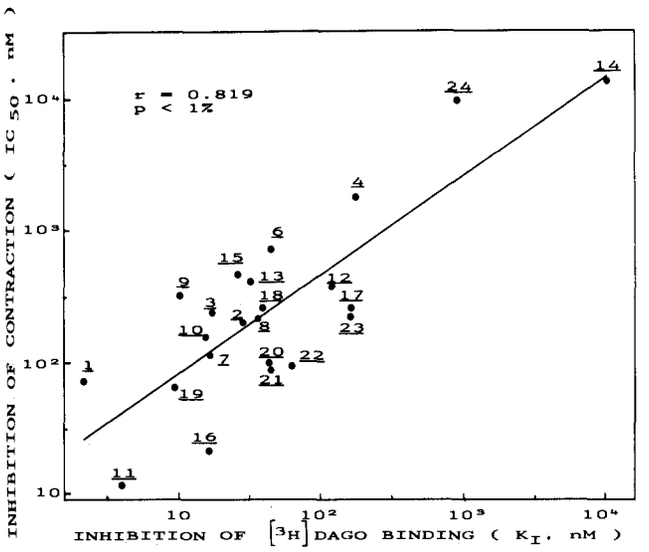


**Figure 1.** Correlation between log potency in the in vitro GPI assay ($y$) and in the [$^3$H]DAGO (μ) receptor binding assay ($x$) was studied by using data from Tables I and II. The regression line is $y = 0.757x + 1.16$.

Extension of the enkephalins by an additional C-terminal hydrophilic moiety as in DSLET or DTLET favors binding to the δ sites. This has been attributed to the importance of this residue for conferring the proper orientation to the aromatic nucleus of Phe$^4$ for optimal interaction with a δ-specific subsite.[13,36]

Accordingly, modifications of the sixth residue were carried out to characterize the structure optimizing δ specificity. A comparison of the activities on the peripheral tests of the hexapeptides **13**, **17** or **15**, and **18** underlines the importance of a hydroxylic group on the C-terminal position. Indeed, in both cases, the replacement of Thr by Leu produced a decrease in δ selectivity. This effect was also observed by removing the terminal carboxylic function (compound **19**). The elimination of the carboxyl group of enkephalins or its transformation to an amide or alcohol has often been reported to increase μ recognition but concomitantly to greatly decrease selectivity (review in ref 37). Likewise, introduction of the amino acids Hyp in **21**, N(Me)Phe in **22**, and N(Me)Leu in **23** was shown to induce a cis–trans isomerism in these peptides (not shown) and a decrease in the affinity for the δ sites. Nevertheless, this effect, which was previously described in the case of [Leu]enkephalin derivatives,[31] contrasts with the data obtained in the Metkephamid series.[34] Therefore, it seems that N-alkylation induces various effects depending on the overall structure of the parent peptide.

**Correlations between Peripheral Activity ($IC_{50}$ on GPI and MVD Assays) and Inhibitory Potency on [$^3$H]DAGO ($K_i$) and [$^3$H]DSLET ($K_i$) Binding in Rat Brain Tissue.** The results obtained with the peptides in the GPI and MVD bioassays are quantitatively in accordance with their binding properties to brain tissue (Table II). This is clearly illustrated in Figure 2 by the linear correlation ($r = 0.931$) between the inhibiting effects in the MVD bioassay of the peptides studied and their apparent affinity for rat brain receptors labeled with [$^3$H]DSLET.

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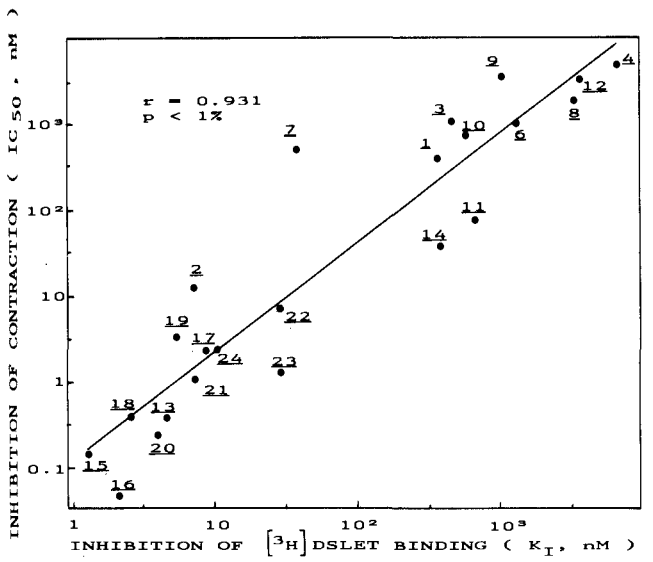


**Figure 2.** Correlation between log potency in the in vitro MVD assay ($y$) and in the [$^3$H]DSTLET ($\delta$) receptor binding assay ($x$) was studied by using data from Tables I and II. The regression line is $y = 1.26x - 0.92$.

The only important discrepancy occurred with compound **7**, which exhibited a pharmacological potency on the MVD weaker than that expected from its affinity for brain $\delta$ receptors. This result remains unexplained, but it is important to realize that there is no basis for assigning the label of partial agonist to **7** since the maximum response induced by this compound on MVD or GPI bioassays was the same as that of [Met]enkephalin.

The correlation was less significant when the ability of the various compounds to inhibit the electrically evoked contractions of the GPI were compared with their affinities for central $\mu$ receptors ([$^3$H]DAGO binding assay) (Figure 1). This could be related to the receptor heterogeneity of the guinea pig ileum preparation.[38] Nevertheless, an important involvement of $\kappa$ receptors in the pharmacological effects of the present peptides is unlikely since these compounds were unable to inhibit the binding of the $\kappa$ ligand [$^3$H]ethylketocyclazocine to rat brain tissue at a concentration as high as 1000 nM. Finally, the overall good agreement between the results of in vitro pharmacological experiments and binding studies indicate that the present compounds are similarly protected from processing enzymes in the various tissues.

**Characterization of the Pharmacological Selectivity by the Receptor Inactivation Approach.** The pharmacological properties of opioid compounds are usually assessed by the GPI and MVD bioassays. These two tissues are considered as specific tests for $\mu$ and $\delta$ agonists, respectively.[39] However, the presence of a small fraction of non-$\mu$ or non-$\delta$ receptors could lead to an imprecise determination of the real potency of a compound toward any pure receptor. To overcome this problem, preparations enriched in a single opioid receptor have been used.[30] Such an approach was used in the present work, through protection of $\delta$ receptors of the MVD by DSLET and DTLET before receptor inactivation by $\beta$-chlornaltrexamine ($\beta$-CNA), a site-directing alkylating agent.[40] Various agonists were tested before and after the inactivation. As shown in Table III, DAGO, a prototype $\mu$ agonist, loses its potency on the MVD, enriched in $\delta$ receptors, to a considerable extent (24-fold). In contrast, $\delta$-selective agonists such as DSLET and DTLET show the smallest potency shifts (1.7). Taken together, these results strongly suggest that the pharmacological response of the MVD to agonists are mediated not only by $\delta$ receptors, but also by $\mu$ receptors. Therefore, DTLET appears to be an appropriate selective ligand for $\delta$ receptors in bioassays preparations as its very high potency seems to be unaffected by alkylation of binding sites different from the $\delta$ type.

**Table III.** Potency Shifts in MVD Assay after Selective Protection from $\beta$-CNA-Induced Inactivation by DTLET of Opioid Receptors[a]

| compound | $IC_{50}$ (nM) before treatment | $IC_{50}$ (nM) after treatment | ratio |
|---|---|---|---|
| **13**, DSLET (6) | 0.38 ± 0.09 | 0.65 ± 0.29 | 1.7 |
| **15**, DTLET (3) | 0.13 ± 0.02 | 0.22 ± 0.10 | 1.7 |
| 11, DAGO (3) | 100 ± 50 | 2400 ± 1000 | 24.0 |

[a] Data are means ± SD. The number of observations is given in parentheses. Ratio is $IC_{50}$ after treatment/$IC_{50}$ before treatment.

**Table IV.** Comparison between Analgesic Potencies in Mice (Hot Plate Test, Icv Administration) of Several Opioid Peptides with Their Abilities To Inhibit the Binding of [$^3$H]DAGO (1 nM) and [$^3$H]DSLET (2 nM) from Rat Brain Tissue[a]

| compound | analgesia ($AD_{50}$, pmol)[b] | $K_i$ [$^3$H]DAGO[c] | $K_i$ [$^3$H]-DSLET[c] |
|---|---|---|---|
| 11, DAGO | 1 (0.5–2.5) | 3.9 ± 0.8 | 700 ± 95 |
| DADLE[a] | 12 (6–24) | 8.4 ± 0.6 | 7.7 ± 0.4 |
| **15**, DTLET | 50 (30–90) | 25.3 ± 2.5 | 1.35 ± 0.15 |
| **13**, DSLET | 130 (60–250) | 31.0 ± 5.0 | 4.8 ± 0.8 |
| **21**, DSLEH | 252 (130–487) | 43.8 ± 8.3 | 7.6 ± 0.4 |
| **17**, DSLEL | 610 (391–952) | 158 ± 9 | 9.1 ± 1.6 |

[a] Tyr-D-Ala-Gly-Phe-D-Leu from ref 5. [b] Analgesia was evaluated by icv injection 10 min before exposing animals to the hot plate. Each point is the mean value obtained with 10 mice. The $AD_{50}$ values are between parentheses, the 95% confidence limits are shown for each line. [c] The $K_i$ values are the means ± SEM of three to six independent determinations in triplicate. Each $K_i$ value was obtained from computer analysis of Hill plots with nine concentrations of unlabeled ligand.

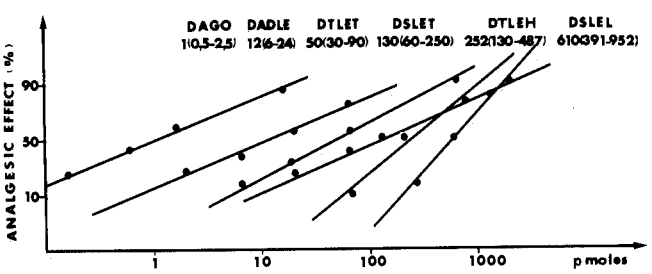


**Figure 3.** Analgesic effects of opioids peptides: DAGO (11), DADLE, DTLET (15), DSLET (13), DSLEH (21), and DSLEL (17). All peptides were injected icv 10 mn before exposing animals to the hot plate. Each point is the mean value obtained with 10 mice. The $AD_{50}$ and, between parentheses, the 95% confidence limits are shown for each line.

**Analgesic Effects of Enkephalin-Related Peptides.** Previous studies[6,7] have shown that for some selective enkephalin analogues, there is a highly significant and positive correlation between their analgesic potency ($AD_{50}$ in the hot plate test) and both their inhibitory effect on the GPI ($IC_{50}$) and their ability to inhibit [$^3$H]DAGO binding ($K_i$) in rat brain tissue.

In the present work, such a study was extended to a large number of compounds. As shown in Figure 3, when icv injected, the opioid peptides and [D-Ala$^2$,D-Leu$^5$]enkephalin display dose-dependent analgesic effects in the hot plate

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test with dose–response lines roughly parallel. The analgesic effects of these latter compounds in the hot plate test ($AD_{50}$) and their $K_i$ values in competition experiments against [$^3$H]DAGO and [$^3$H]DTLET are reported in Table IV. The results show that the antinociceptive activity of these peptides is related to their affinity for brain μ opioid binding sites. This is consistent with the often expressed view that the analgesic effect of exogenous or endogenous opioid peptides is preferentially ensured through μ-receptor activation, at least at the supraspinal level.[5,7,8]

## Conclusion

Modifications of the sequence of enkephalins, following the previous proposed rules for selective μ or δ recognition,[13] led to a large number of μ and δ agonists belonging to two series of chemically related peptides that display a wide range of affinities. These compounds could be useful to investigate the physiological role of μ and especially δ[9,12] opioid receptors in the central nervous system. Furthermore, the interesting shift toward δ selectivity observed after introduction of a *tert*-butyl group in position 2 in the TRIMU series prompted us to test this type of modification in the sequence of DSLET and DTLET (in preparation).

## Experimental Section

**Chemistry.** [Met]enkephalin, DAGO, Tyr-D-Pen-Gly-Phe-Pen and Tyr-D-Pen-Gly-Phe-D-Pen were from Bachem AG. Their analytical data sheet show that their purity is assessed by TLC (two systems) and by HPLC (purity >99%). The peptide content is quantitated by quantitative amino acid analysis (>95%). The peptides were prepared from protected amino acids (Bachem AG) by the liquid-phase method with *tert*-butyloxycarbonyl, benzyloxycarbonyl, and methyl and benzyl esters as protecting groups and dicyclohexylcarbodiimide with hydroxybenzotriazole as coupling reagent.[41]

The structure and the lack of racemization of the compounds and all of the intermediates were established by $^1$H NMR spectroscopy (Bruker WH, 270 and 400 MHz). Complete assignment of $^1$H NMR signals of all the products was performed by classical double resonance experiments already described for the enkephalins.[42,43] Chemical shifts (in parts per million ± 0.02) relative to HMDS as an internal reference were reported only for the most relevant compounds. The purity was checked by thin-layer chromatography on (Merck) silica gel plates in the following solvent systems (v/v):A, chloroform–methanol (9:1); B, BuOH–AcOH–$H_2O$ (4:1:1); C, chloroform–methanol (7:3); D, chloroform–methanol(15:1). The products were also tested by HPLC (Waters apparatus) on a reverse-phase μ Bondapak $C_{18}$ column with $NH_4OAc$, $10^{-2}$ M buffer (pH 4.2) –$CH_3CN$ as solvents. The eluted peaks were monitored at 210 nm.

Melting points of the crystallized products are reported uncorrected.

Analyses are given for the final compounds, except for the trifluoroacetate salts, which are too highly hygroscopic. In these cases, analyses are given for the parent compounds and retention times on HPLC are reported for the trifluoroacetate salts.

Amino acid analysis was carried out on an LKB Biochrom 4400 analyzer after hydrolysis by 6 N HCl, at 110 °C for 24 h. Mass spectra were recorded on a double-focusing VG 70-250 instrument. The FAB ionization was obtained with a FAB saddle field source (Ion Tech Ltd, Teddington, UK) operated with xenon at 8 kV and 1 mA. Glycerol or cesium iodide was used for calibration. Accelerating voltage was set at 6 kV, and resolution was 1200. Mass spectra were obtained in different matrices and processed by means of the VG-250 software package.

[$^3$H]DAGO (2.2 TBq/mmol) was from CEA, Gif-sur-Yvette, France, and [$^3$H]DSLET (1.1 TBq/mmol) from NEN. Levorphanol (tartrate) was a generous gift from Hoffmann-La Roche.

**Preparation of Membrane Fraction and Binding Assays.** Membrane fraction was prepared as previously described.[40] Binding studies were carried out in 50 mM Tris·HCl (pH 7.4). Each assay contained 0.6–0.7 mg of protein, the radioligand ([$^3$H]DAGO, 1 nM; [$^3$H]DSLET, 2 nM), and other additions in a final volume of 1 mL. All points were determined in triplicate, and the nonspecific binding was measured in the presence of 10 μM levorphanol. After a 40-min incubation at 35 °C, the contents of each tube were rapidly filtered over Whatman GF/B filters. The filters were washed twice with 5 mL of cold Tris·HCl buffer, dried, and suspended in 5 mL of scintillation liquid (Beckman Ready Solv. EP cocktail) to determine bound radioactivity (counting efficiency ≃ 40%).

**Data Analysis.** The specific binding of tritiated ligands was defined as the difference in the means of triplicate determinations of the binding, measured in the presence and the absence of 10 μM levorphanol. Competition curves were fitted by linear regression analysis of the Hill transformation. $K_i$ values were calculated according to the Cheng–Prusoff relationship,[45] assuming competitive interactions.

**Biological Tests.** The compounds were assayed for their μ and δ opioid activities on the GPI and MVD, respectively, as previously described.[39] The agonist concentration that produces half-maximal inhibition of the electrically stimulated muscle twitch, $IC_{50}$, was determined from six to eight computed log dose–response curves with six different concentrations of the compound. Since the sensitivity varied from one tissue to another, [Met]enkephalin $IC_{50}$ was determined on the same preparation, and the agonist $IC_{50}$ was corrected by multiplication of the ratio: mean of all the [Met]enkephalin $IC_{50}$/[Met]enkephalin $IC_{50}$ of the assay.

**Receptor Inactivation and Pharmacological Assays.** Vasa deferentia of 35–45-g mice (Deprē, France) were dissected and set up for electrical stimulation as previously described.[41] The DTLET concentration used to protect δ receptors as 1 μM. Receptor inactivation was carried out by adding β-CNA to the bath at a final concentration of 0.1 μM. After an incubation of 30 min at 37 °C, the preparation was washed 15 times in 30 min to remove excess DTLET and β-CNA. Finally, the electrical stimulation was applied, and the compound to be tested was added. The agonist concentration that produced half-maximal inhibition of the electrically stimulated muscle twitch, $IC_{50}$, was computed from log dose–response curves. Since the tissue sensitivity varied, [Met]enkephalin $IC_{50}$ was determined on each preparation before inactivation, and the agonist $IC_{50}$ was corrected by multiplication of the ratio: mean of all the [Met]enkephalin $IC_{50}$/[Met]enkephalin $IC_{50}$ of the assay.

**Analgesic Tests.** Analgesic potencies were evaluated on the hot plate test at 55 ± 0.2 °C with albino mice (Deprē, France) weighing 24–28 g.[7] The hot plate was surrounded by a cylindrical plexiglass chimney (14 cm diameter × 20 cm high). The jump latency time was measured by means of a stopwatch 10 min after intracerebroventricular (icv) drug administration by hand (10-μL volume) with a modified 30-gauge needle and a Hamilton syringe.[46] Mice that did not jump within 180 s were removed (cut-off time). The experimenter was blind with respect to pretest manipulations. The results were expressed as a percentage of analgesia according to the formula $T_t - T_c/180\text{ s} - T_c$ ($T_t$ and $T_c$ are the jump latency times of treated and control animals, respectively). The cut-off time (180 s) represents 100% analgesia.

**Statistical Analysis.** Each value was the mean of measures obtained with 10 mice. The $AD_{50}$ and their 95% confidence limits were determined according to the method of Litchfield and Wilcoxon.[47]

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***N*-(*tert*-Butyloxycarbonyl)-L-tyrosyl-α-aminoisobutyric Acid Methyl Ester.** To a solution of *N*-(*tert*-butyloxycarboxyl)-L-tyrosine (2.81 g, 10 mmol) in anhydrous THF (15 mL) cooled at 0 °C were added successively a solution of α-aminoisobutyric acid methyl ester hydrochloride (1.53 g, 10 mmol) and triethylamine (1.4 mL) in $CHCl_3$ (15 mL), a solution of HOBT (1.53 g, 10 mmol) in THF (10 mL), a solution of DCC (2.06 g, 10 mmol) in $CHCl_3$ (10 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 (30 mL) and washed successively with a saturated solution of NaCl (25 mL), a 10% solution of citric acid (4 × 25 mL), water (25 mL), a 10% solution of $NaHCO_3$ (3 × 25 mL), and, finally, a satured solution of NaCl (25 mL). The solvent was dried in $Na_2SO_4$ and evaporated in vacuo. *This procedure is designated as the standard treatment.* The protected dipeptide was obtained as a white solid and recrystallized from EtOAc: yield 3.30 g (87%); TLC $R_f$ (A) 0.54; mp 82–84 °C; FAB-MS ($MH^+$) calcd 381, found 381.

***N*-(*tert*-Butyloxycarbonyl)-L-tyrosyl-α-aminoisobutyrylglycine Methyl Ester.** To a solution of the preceding compound (1.89, 5 mmol) in MeOH (10 mL) cooled at 0 °C was added 10 mL of 1 N NaOH. 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 1.65 g (90%) of the pure protected tripeptide: mp 158–160 °C; TLC $R_f$ (B) 0.95.

***N*-(*tert*-Butyloxycarbonyl)-L-tyrosyl-α-aminoisobutyrylglycine Methyl Ester.** To a solution of *N*-(*tert*-butyloxycarbonyl)-L-tyrosyl-α-aminoisobutyric acid (1.46 g, 4 mmol) in anhydrous THF (10 mL) cooled in an ice–water bath were added successively a mixture of glycine methyl ester hydrochloride (0.51 g, 4 mmol) and triethylamine (0.56 mL) in $CHCl_3$ (20 mL), a solution of HOBT (0.61 g, 4 mmol) in anhydrous THF (10 mL), and a solution of DCC (0.82 g, 4 mmol) in $CHCl_3$ (10 mL). After 1 h at 0 °C, the mixture was allowed to come to room temperature and stirred for 24 h. The reaction was worked up following the standard treatment and yielded a white solid, 1.31 g (75%): mp 136–138 °C; TLC $R_f$ (A) 0.45; FAB-MS ($MH^+$) calcd 438, found 438.

**(*R,S*)-*N*-(*tert*-Butyloxycarbonyl)-L-tyrosyl-α-aminoisobutyrylglycine 1,3-Dimethylbutylamide.** To a solution of the preceding compound (0.876 g, 2 mmol) was added 10 mL of (*R,S*)-1,3-dimethylbutylamine.[5] The mixture was stirred at room temperature for 1 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 0.99 g (98%): mp 98–100 °C; $R_f$ (A) 0.40. Anal. Calcd for ($C_{26}H_{42}N_4O_6$) C, H, N.

**(*R,S*)-L-Tyrosyl-α-aminoisobutyrylglycine 1,3-Dimethylbutylamide Trifluoroacetate (4).** The preceding compound (0.51 g, 1 mmol) was dissolved in TFA (1.5 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 (150 mL) led to the precipitation of crude compound **4**. The white solid was washed with ether (5 × 80 mL) and dried in vacuo: yield 0.45 g (87%); TLC $R_f$ (B) 0.66; $R_f$ (C) 0.50; HPLC (reversed-phase μ Bondapak $C_{18}$), solvent $NH_4AcO$ buffer $10^{-2}$ M pH 4.2/$CH_3CN$ (60:40), flow rate 1.2 mL/min, retention time 5.1 min; FAB-MS ($MH^+$) calcd 507, found 507. Amino acid anal.: Tyr 1.00 (1.0), Gly 1.01 (1.0).

***N*-(*tert*-Butyloxycarbonyl)-L-tyrosyl-*O*-*tert*-butyl-D-serylglycine Methyl Ester.** To a solution of *N*-(*tert*-butyloxycarbonyl)-L-tyrosyl-*O*-*tert*-butyl-D-serine[5] (0.85 g, 2 nmol) in anhydrous THF (20 mL) were added successively a mixture of glycine methyl ester hydrochloride (0.25 g, 2 mmol) and triethylamine (0.28 mL) in $CHCl_3$ (20 mL), a solution of HOBT (0.31 g, 2 mmol) in anhydrous THF (10 mL), and a solution of DCC (0.41 g, 2 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: 0.86 g (87%); mp 108–110 °C; TLC $R_f$ (A) 0.60; FAB-MS ($MH^+$) calcd 496, found 496. Anal. Calcd for ($C_{25}H_{40}N_4O_7$) C, H, N.

**(*R,S*)-L-Tyrosyl-*O*-*tert*-butyl-D-serylglycine 1,3-Dimethylbutylamide (6) and (*R,S*)-L-Tyrosyl-D-serylglycine 1,3-Dimethylbutylamide (7).** To a solution of the preceding compound (0.43 g 0.87 mmol) in 5 mL of MeOH was added 10 mL of (*R,S*)-1,3-dimethylbutylamine.[5] The reaction was stirred for 1 week, and the solvent and excess of amine were removed in vacuo. The residue was triturated with ether, and the white solid obtained was washed with ether and dried. Then, the compound was dissolved in a mixture of TFA/$CH_2Cl_2$, 50:50, (1.30 mL) at 0 °C. After 20 min at 0 °C, the addition of ether (80 mL) led to the precipitation of a white solid, which was washed with ether (5 × 80 mL) and dried in vacuo to yield 0.410 g of a mixture of two products, which were separated on a silica gel column with $CHCl_3$/MeOH (7:3) as eluent. The first compound eluated was identified as **6** (0.18 g, 40%); TLC $R_f$ (B) 0.70, $R_f$ (C) 0.52 and the second one as **7** (0.14 g, 35%), TLC $R_f$ (B) 0.40, $R_f$ (C) 0.28: HPLC (μ Bondapak $C_{18}$), $NH_4OAc/CH_3CN$ (60:40), 1.2 mL/min, retention time of **6**, 8.2 min, and retention time of **7**, 3.9 min.

**6**: NMR (MeSO-$d_6$) δ 4.03 (Tyr α-H), 2.84 (β-$CH_2$), 6.61 and 7.05 (Ar H), 4.40 (Ser α-H), 3.25 (β-$CH_2$), 3.60 (Gly $CH_2$), chain protons δ 3.80 [CH(NH)], 0.95 ($CH_3$), 1.11 and 1.24 ($CH_2$), 1.49 [CH(isopropyl)], 0.79 [$CH_3$(isopropyl)].

**7**: NMR ($Me_2SO$-$d_6$) δ 3.93 (Tyr α-H), 2.76 and 2.90 (β-$CH_2$), 6.56 and 6.97 (Ar H), 4.20 (Ser α-H), 3.25 and 3.40 (β-$CH_2$), 1.05 (*t*-Bu), 3.56 (Gly $CH_2$), chain protons δ 3.75 [CH(NH)], 0.91 ($CH_3$), 1.05 and 1.18 ($CH_2$), 1.44 [CH(isopropyl)], 0.78 [$CH_3$(isopropyl)].

Amino acid anal. **6**: Tyr 1.01 (1.0), Ser 0.98 (1.0), Gly 1.04 (1.0). Amino acid anal. **7**: Tyr 1.0 (1.0), Ser 0.95 (1.0), Gly 1.03 (1.0).

***N*-(*tert*-Butyloxycarbonyl)-L-tyrosyl-D-alanylglycine.** To a solution of *N*-(*tert*-butyloxycarbonyl)-L-tyrosyl-D-alanylglycine methyl ester[5] (0.33 g, 0.78 mmol) in MeOH (5 mL) was added 1.6 mL of 1 N NaOH at 0 °C. The mixture was stirred at 0 °C for 30 min and then at room temperature for 5 h. The reaction was treated following the standard procedure for alkaline hydrolysis and produced a white solid: yield 0.26 g (81%); mp 146–148 °C; TLC $R_f$ (B) 0.68. Amino acid anal.: Tyr 1.02 (1.0), Ala 0.95 (1.0).

**(*R,S*)-*N*-(*tert*-Butyloxycarbonyl)-L-tyrosyl-D-alanylglycine *N*-Ethyl 1,3-Dimethylbutylamide.** To a solution of *N*-(*tert*-butyloxycarbonyl)-L-tyrosyl-D-alanylglycine (0.41 g, 1 mmol) in anhydrous THF (5 mL) were added successively a solution of *N*-ethyl-1,3-dimethylbutylamine (0.13 g, 1 mmol) in THF (5 mL), a solution of HOBT (0.15 g, 1 mmol) in THF (2.5 mL), and a solution of DCC (0.21 g, 1 mmol) in $CHCl_3$ (2.5 mL). After 1 h at 0 °C, the mixture was allowed to come to room temperature and then stirred for 36 h. The reaction was treated following the standard procedure, and the solid obtained was purified by flash chromatography on silica gel with $CHCl_3$/MeOH (20:1) as eluent. Fractions containing pure peptide were evaporated in vacuo to yield 0.28 g (54%): mp 112–114 °C; TLC $R_f$ (A) 0.40. Anal. Calcd for ($C_{27}H_{44}N_4O_6$) C, H, N.

**(*R,S*)-L-Tyrosyl-D-alanylglycine *N*-Ethyl-1,3-dimethylbutylamide (8).** As for compound **5**, the *N*-*tert*-butyloxycarbonyl group was removed by TFA. From 86 mg (0.17 mmol) of the preceding compound, 76 mg (86%) of **7** was obtained as a white solid: TLC $R_f$(B) 0.45, $R_f$ (C) 0.39; HPLC (μ Bondapak $C_{18}$), $NH_4OAc/CH_3CN$ (60:40), 1.2 mL/min, retention time 5.2 mn; FAB-MS ($MH^+$) calcd 521, found 521. Amino acid anal.: Tyr 0.95 (1.0), Ala 1.01 (1.0), Gly 1.00 (1.0).

**(*R,S*)-*N*-(*tert*-Butyloxycarbonyl)-L-tyrosyl-D-alanylglycine 3-Methylbutylamide.** To a solution of *N*-(*tert*-butyloxycarbonyl)-L-tyrosyl-D-alanylglycine methyl ester (0.42 g, 1 mmol) in MeOH (5 mL) was added 3.5 mL of 3-methylbutylamine at 0 °C. After 30 min, the mixture was allowed to come to room temperature and stirred overnight. After removal of both the solvent and the excess of amine, the residue was triturated with ether to obtain a white solid. The product weighed 0.48 g (99%): mp 108–110 °C; $R_f$ (A) 0.20. Anal. Calcd for ($C_{24}H_{38}N_4O_6$) C, H, N.

**(*R,S*)-L-Tyrosyl-D-alanylglycine 3-Methylbutylamide (9) (TRIMU 5).** As for compound 4, the *N*-*tert*-butyloxycarbonyl

(47) Litchfield, J. T.; Wilcoxon, F. *J. Pharmacol. Exp. Ther.* **1949**, *96*, 99.

group was removed by TFA. From 970 mg of the preceding compound, 240 mg (89%) of 8 was obtained: TLC $R_f$ (B) 0.32, $R_f$ (C) 0.22; HPLC (μ Bondapak $C_{18}$, $NH_4Ac/CH_3CN$ (60:40), 1.2 mL/min, retention time 4 min; NMR ($Me_2SO$-$d_6$) δ 3.95 (Tyr α-H), 2.81 (β-$CH_2$), 6.60 and 6.99 (Ar H), 4.30 (Ala α-H), 1.05 ($CH_3$), 3.57 (Gly $CH_2$), chain protons δ 3.05 [$CH_2$(NH)], 1.25 ($CH_2$), 1.50 [CH(isopropyl)], 0.80 [$CH_3$(isopropyl)]; FAB-MS ($MH^+$) calcd 479, found 479. Amino acid anal.: Tyr 0.96 (1.0), Ala 1.00 (1.0), Gly 0.94 (1.0).

**(*R*,*S*)-*N*-(*tert*-Butyloxycarbonyl)-L-tyrosyl-D-methionylglycine 3-Methylbutylamide.** To a solution of *N*-(*tert*-butyloxycarbonyl)-L-tyrosyl-D-methionylglycine methyl ester[5] (0.67 g, 1.39 mmol) was added 3.5 mL of 3-methylbutylamine at 0 °C. After 30 min, the mixture was allowed to come to room temperature and stirred overnight. After evaporation and treatment with ether, a white solid was obtained: yield 0.76 g (99%); mp 92–93 °C; TLC $R_f$ (A) 0.42. Anal. Calcd for ($C_{26}H_{42}N_4O_6S$) C, H, N, S.

**(*R*,*S*)-L-Tyrosyl-D-methionylglycine 3-Methylbutylamide (10).** As for compound 4, the *N*-*tert*-butyloxycarbonyl group was removed by TFA. From 0.76 g (1.4 mmol) of the preceding compound, 0.70 g (90%) of **9** was obtained: TLC $R_f$ (B) 0.44, $R_f$ (C) 0.35; FAB-MS ($MH^+$) calcd 539, found 539. Amino acid anal.: Tyr 0.95 (1.0), Ala 1.00 (1.0), Gly 0.94 (1.0).

***N*-(*tert*-Butyloxycarbonyl)-L-tyrosyl-D-alanylglycyl-*N*-methyl-L-leucylglycinol.** To a solution of *N*-(*tert*-butyloxycarbonyl)-L-tyrosyl-D-alanylglycine (0.41 g, 1 mmol) in anhydrous THF (10 mL), cooled in an ice–water bath, were successively added a mixture of *N*-methyl-L-leucine benzyl ester *p*-tosylate (0.41 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 (0.21 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 overnight. The reaction was treated following the standard procedure, and a solid was obtained: 0.42 g (70%); $R_f$ (A) 0.75.

To a solution of the preceding compound (420 mg, 0.7 mmol) in MeOH (5 mL) was added 1.4 mL of 1 N NaOH at 0 °C. The mixture was stirred for 1 h at 0 °C and then at room temperature overnight. The reaction was treated following the standard procedure for alkaline hydrolysis and yielded 320 mg (85%) of the N-protected pentapeptide. To this latter compound, dissolved in THF (10 mL), were added successively a solution of ethanolamine (0.035 mL) in $CHCl_3$ (5 mL), a solution of HOBT (89 mg 0.58 mmol) in THF (5 mL), and a solution of DCC (120 mg, 0.58 mmol) in $CHCl_3$ (5 mL). After 1 h at 0 °C, the mixture was stirred at room temperature for 20 h. The reaction was worked up following the standard treatment, and the crude product was obtained; 200 mg was purified by flash chromatography on silica gel with $CHCl_3$/MeOH (15:1) as eluent. Fractions containing pure N-protected pentapeptide were evaporated in vacuo to yield 160 mg (50%): mp 158–160 °C; TLC $R_f$ (D) 0.30; FAB-MS ($MH^+$) calcd 815, found 815.

**L-Tyrosyl-D-alanylglycyl-*N*-methyl-L-leucylglycinol (12).** As for compound **5**, the *N*-*tert*-butyloxycarbonyl group was removed by TFA. From 40 mg (0.07 mmol) of the preceding compound, 30 mg of **11** (75%) was obtained as a white solid: TLC $R_f$ (B) 0.27, $R_f$ (C) 0.21; FAB-MS ($MH^+$) calcd 715, found 715. Anal. Calcd for ($C_{23}H_{37}N_5O_6$) C, H, N.

***N*-(*tert*-Butyloxycarbonyl)-L-tyrosyl-*O*-*tert*-butyl-D-serylglycyl-L-phenylalanyl-L-leucyl-L-leucine Methyl Ester.** To a solution of *N*-(*tert*-butyloxycarbonyl)-L-tyrosyl-*O*-*tert*-butyl-D-serylglycyl-L-phenylalanyl-L-leucine (1.48 g, 2 mmol) in THF (15 mL), cooled in an ice–water bath, were added successively a solution of L-leucine methyl ester hydrochloride (0.36 g, 2 mmol) and triethylamine (0.28 mL) in $CHCl_3$ (15 mL), a solution of HOBT (0.31 g, 2 mmol) in THF (10 mL), and a solution of DCC (0.41 g, 2 mmol) in $CHCl_3$ (10 mL). After 1 h at 0 °C, the mixture was stirred for 20 h at room temperature. The reaction was then treated following the standard procedure, and a white solid (1.60 g, 90%) was obtained, which showed a single spot on TLC $R_f$ (A) 0.65, mp 164–166 °C; FAB-MS ($MH^+$) calcd 870, found 870.

**L-Tyrosyl-D-serylglycyl-L-phenylalanyl-L-leucyl-L-leucine (17).** To a solution of the preceding compound (1.60 g, 1.8 mmol) in MeOH cooled at 0 °C was added 4 mL of 1 N NaOH. The mixture was stirred at room temperature for 24 h. The reaction mixture was treated following the standard procedure for alkaline hydrolysis and yielded the N-protected hexapeptide. The preceding compound was dissolved at 0 °C in TFA saturated with HCl (5 mL). The reaction mixture was stirred at 0 °C for 30 min and then at room temperature for 30 min. The classical treatment (see compound **5**) led to a crude product, which was purified by silica gel filtration on LH 20 (Pharmacia) with MeOH as eluent. Fractions containing the pure hexapeptide were evaporated and lyophilized to yield 0.89 g (65%): TLC $R_f$ (B) 0.77, $R_f$ (C) 0.55; mp 137–139 °C; FAB-MS ($MH^+$) calcd 699, found 699. Anal. Calcd for ($C_{35}H_{50}N_6O_9$) C, H, N.

**Tyrosyl-D-serylglycyl-L-phenylalanyl-L-leucyldecarboxythreonine (19).** To a solution of *N*-(*tert*-butyloxycarbonyl)-L-tyrosyl-*O*-*tert*-butyl-D-serylglycyl-L-phenylalanyl-L-leucine methyl ester hydrochloride (0.68 g, 0.9 mmol) in DMF (10 mL) cooled at 0 °C was added 4.16 g (54 mmol) of (*R*)-1-amino-2-propanol. Then, the mixture was allowed to come to room temperature and stirred for 1 week. The reaction was diluted with water (10 mL), acidified with 1 N HCl (0.5 mL), and extracted with EtOAc (3 × 30 mL). The organic layer was washed with $H_2O$ (20 mL) and a saturated solution of NaCl. The solvent was dried and evaporated in vacuo: yield 0.67 g (90%); mp 122–124 °C; TLC $R_f$ (B) 0.14.

A sample of the preceding compound (80 mg, 0.10 mmol) was deprotected and purified as described for **17**: yield 55 mg (85%) of pure hexapeptide **19**; mp 140–142 °C; TLC $R_f$ (B) 0.38, *Rf* (C) 0.25; FAB-MS ($MH^+$) calcd 687, found 687. Anal. Calcd for ($C_{32}H_{46}N_6O_8$) C, H, N.

***N*-(Benzyloxycarbonyl)-L-tyrosyl-D-threonylglycyl-L-phenylalanyl-L-leucyl-L-threonine Benzyl Ester.** To a solution of *N*-(benzyloxycarbonyl)-L-tyrosine (1.26 g, 4 mmol) in THF (15 mL), cooled at 0 °C, were added successively a solution of D-threonine benzyl ester hemioxalate (1.20 g, 4 mmol) and triethylamine (0.56 mL) in $CHCl_3$ (15 mL), a solution of HOBT (0.61 g, 4 mmol) in THF (10 mL), and a solution of DCC (0.82 g, 4 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 1.52 g (75%); mp 85–87 °C; TLC $R_f$ (A) 0.51. To a solution of the preceding compound (1.04 g, 2 mmol) in MeOH (5 mL) was added 4 mL of 1 N NaOH, and the mixture was stirred at 0 °C for 1 h and at room temperature for 6 h. The reaction was treated following the standard procedure and produced a white solid: yield 0.67 g (80%); mp 70 °C; TLC $R_f$ (B) 0.79.

To a solution of *N*-(benzyloxycarbonyl)-L-tyrosyl-D-threonine (0.22 g, 0.5 mmol) in THF (5 mL), cooled at 0 °C, were added successively a solution of glycyl-L-phenylalanyl-L-leucyl-L-threonine benzyl ester trifluoroacetate[48] (0.32 g, 0.5 mmol) and triethylamine (0.07 mL) in $CHCl_3$ (5 mL), a solution of HOBT (0.76 g, 0.5 mmol) in THF (2.5 mL), and a solution of DCC (0.11 g, 0.5 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 as usual, and the crude product was obtained and purified by flash chromatography on Kieselgel 60 with $CHCl_3$/MeOH (20:1) as eluent. Fractions containing pure diprotected hexapeptide were collected: yield 0.32 g (70%); mp 154–156 °C; TLC $R_f$ (D) 0.32; FAB-MS ($MH^+$) calcd 926, found 926.

**L-Tyrosyl-D-threonylglycyl-L-phenylalanyl-L-leucyl-L-threonine (15).** A solution of the preceding compound (100 mg, 0.14 mmol) in 5 mL of MeOH containing 4 drops of AcOH was hydrogenated at room temperature and atmospheric pressure over Pd/C (10%, 15 mg) during 4 h. After removal of the catalyst, the solution was evaporated to dryness, yielding 70 mg (92%) of the pure hexapeptide. The purity of the hexapeptide was checked both by TLC (single spot, $R_f$ (B) 0.33, $R_f$ (C) 0.20) and by HPLC on a Waters apparatus (μ Bondapack C18), $NH_4^+$ $AcO^-$ buffer $10^{-2}$ M pH 4.2/$CH_3CN$ (75:25), 1.2 mL/min, retention time of single peak 5.2 min; FAB-MS ($MH^+$) calcd 702, found 702. Anal. Calcd for ($C_{34}H_{48}N_6O_{10}$) C, H, N.

***N*-(*tert*-Butyloxycarbonyl)-glycyl-*N*-methyl-L-phenylalanyl-L-leucine Methyl Ester.** To a solution of *N*-(*tert*-butyloxycarbonyl)-*N*-methyl-L-phenylalanine (1.67 g, 6 mmol) in

(48) Gacel, G.; Dodey, P.; Roques, B. P.; Morgat, J. L.; Roy, J.; Fromageot, P. *J. Labeled Compd. Radiopharm.* **1983**, *20*, 719.

THF (45 mL), cooled at 0 °C, were added successively a solution of leucine methyl ester hydrochloride (1.09 g, 6 mmol) and triethylamine (0.85 mL) in $CHCl_3$ (45 mL), a solution of HOBT (0.92 g, 6 mmol) in THF (30 mL), and a solution of DCC (1.24 g, 6 mmol) in $CHCl_3$ (30 mL). After 1 h at 0 °C, the mixture was allowed to come to room temperature and stirred overnight. The reaction was treated as usual, and an oily residue was obtained: 1.95 g (80%); TLC $R_f$ (C) 0.87. The preceding compound (1.1 g, 2.71 mmol) was dissolved in TFA (4 mL) 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 1 g (90%) of a white powder, TLC $R_f$ (B) 0.4. Then, the crude compound was dissolved in $CHCl_3$ (40 mL), and to this solution cooled at 0 °C were added successively triethylamine (0.34 mL), a solution of *N*-(*tert*-butyloxycarbonyl) glycine (0.42 g, 2.4 mmol) in THF (30 mL), a solution of HOBT (0.37 g, 2.4 mmol), and a solution of DCC (0.49 g, 2.4 mmol). After 1 h at 0 °C, the mixture was allowed to come to room temperature and stirred overnight. The reaction was treated as usual, and an oily residue was obtained: 1.1 g (92%); TLC $R_f$ (A) 0.80; FAB-MS ($MH^+$) calcd 465, found 465.

***N*-(Benzyloxycarbonyl)-L-tyrosyl-D-threonylglycyl-*N*-methyl-L-phenylalanyl-L-leucyl-L-threonine Benzyl Ester.** To a solution of the preceding compound (1 g, 2.2 mmol) in MeOH cooled at 0 °C was added 4.4 mL of 1 N NaOH. The mixture was stirred at room temperature for 4 h. The reaction was treated following the standard procedure for alkaline hydrolysis and yielded 0.81 g (80%) of the N-protected hexapeptide. This compound was then dissolved in THF (5 mL) at 0 °C, and to this were added successively a solution of threonine benzyl ester hemioxalate (0.54 g, 1.8 mmol) and triethylamine (0.25 mL) in $CHCl_3$ (5 mL), a solution of HOBT (0.28 g, 1.8 mmol) in THF (2 mL), and a solution of DCC (0.37 g, 1.8 mmol) in $CHCl_3$ (2 mL). After 1 h at 0 °C, the reaction was stirred overnight at room temperature. The usual treatment led to a white solid: 0.98 g (85%); mp 60 °C; TLC $R_f$ (A) 0.65.

A sample of the preceding compound (640 mg, 1 mmol) was deprotected as described for **15**, yield 292 mg (44%). To a solution of this compound (261 mg, 0.4 mmol) and triethylamine (55 μL) in $CHCl_3$ (3 mL) were added successively, at 0 °C, a solution of *N*-(benzyloxycarbonyl)-L-tyrosyl-D-threonine (166 mg, 0.4 mmol) in THF (3 mL) and a solution of DCC (90 mg, 0.44 mmol). After 1 h at 0 °C, the reaction mixture was stirred overnight at room temperature. The reaction was treated as usual, and a crude product was obtained and purified by flash chromatography on Kieselgel 60 with $CHCl_3$/MeOH (25:1) as eluent. Fractions containing pure diprotected hexapeptide were collected: yield 262 mg (70%); mp 122–124 °C; TLC $R_f$ (A) 0.43; FAB-MS ($MH^+$) calcd 940, found 940.

**L-Tyrosyl-D-threonylglycyl-*N*-methyl-L-phenylalanyl-L-leucyl-L-threonine (22).** A solution of the preceding compound (70 mg, 0.07 mmol) was hydrogenated over Pd/C (10%, 10 mg) as described for **15**, yielding 50 mg (97%) of the pure hexapeptide: mp 172–174 °C; TLC $R_f$ (B) 0.34, $R_f$ (C) 0.23; FAB-MS ($MH^+$) calcd 716, found 716. Anal. Calcd for ($C_{35}H_{50}N_6O_{10}$) C, H, N.

***N*-(*tert*-Butyloxycarbonyl)-glycyl-L-phenylalanyl-*N*-methyl-L-leucyl-L-threonine Benzyl Ester.** To a solution of *N*-(*tert*-butyloxycarbonyl)glycyl-L-phenylalanine (322 mg, 1 mmol) in THF (10 mL), cooled at 0 °C, were added successively a solution of *N*-methyl-L-leucine benzyl ester *p*-tosylate (407 mg, 1 mmol) and triethylamine (0.14 mL) in $CHCl_3$ (10 mL), a solution of HOBT (153 mg, 1 mmol) in THF (5 mL), and a solution of DCC (206 mg, 1 mmol) in $CHCl_3$ (5 mL). After 1 h at 0 °C, the mixture was stirred at room temperature overnight. The reaction was treated as usual, and an oily residue was obtained: 410 mg (76%); TLC $R_f$ (A) 0.62.

To a solution of the preceding compound (410 mg, 0.76 mmol) in MeOH, cooled at 0 °C, was added 1.6 mL of 1 N NaOH. The mixture was stirred at room temperature for 8 h and then treated following the standard procedure for alkaline hydrolysis and yielded 280 mg (82%) of the N-protected tripeptide. A sample of this compound (225 mg, 0.5 mmol) was dissolved in THF (10 mL). To this solution were added successively a solution of L-threonine benzyl ester, hemioxalate (150 mg, 0.5 mmol), and triethylamine (0.07 mL) in $CHCl_3$ (10 mL), a solution of HOBT (76.5 mg, 0.5 mmol), and a solution of DCC (103 mg, 0.5 mmol). After 1 h at 0 °C, the mixture was stirred at room temperature overnight. The reaction was treated as usual, and a white solid was obtained: yield 210 mg (65%); mp 66–68 °C; TLC $R_f$ (A) 0.72.

***N*-(Benzyloxycarbonyl)-L-tyrosyl-D-threonylglycyl-L-phenylalanyl-*N*-methyl-L-leucyl-L-threonine Benzyl Ester.** A sample of the preceding compound (200 mg, 0.31 mmol) was deprotected as described for **5**, yield 196 mg (97%). To a solution of this compound (196 mg, 0.3 mmol) and triethylamine (42 μL) in $CHCl_3$ (10 mL) were added successively, at 0 °C, a solution of *N*-(benzyloxycarbonyl)-L-tyrosyl-D-threonine (125 mg, 0.3 mmol) in THF (3 mL), a solution of HOBT (46 mg, 0.3 mmol) in THF (5 mL), and a solution of DCC (62 mg, 0.3 mmol) in $CHCl_3$ (5 mL). After 1 h at 0 °C, the reaction mixture was stirred overnight at room temperature. The reaction was then treated as usual and the pure diprotected hexapeptide was obtained: yield 240 mg (97%); mp 126–128 °C; TLC $R_f$ (A) 0.44; FAB-MS ($MH^+$) calcd 940, found 940.

**L-Tyrosyl-D-threonylglycyl-L-phenylalanyl-*N*-methyl-L-leucyl-L-threonine (23).** A solution of the preceding compound (65 mg, 0.07 mmol) was hydrogenated over Pd/C (10%, 20 mg) as described for **15**, yielding 40 mg (80%) of the pure hexapeptide: mp 176–178 °C; TLC $R_f$ (B) 0.63, $R_f$ (C) 0.50; FAB-MS ($MH^+$) calcd 716, found 716. Anal. Calcd for ($C_{35}H_{50}N_6O_{10}$) C, H, N.

***N*-(Benzyloxycarbonyl)-L-tyrosyl-D-threonylglycyl-L-phenylalanyl-L-leucyl Methyl Ester.** To a solution of *N*-(benzyloxycarbonyl)-L-tyrosine-D-threonine (440 mg, 0.9 mmol) in THF (5 mL), cooled at 0 °C, were added successively a solution of glycyl-L-phenylalanyl-L-leucine methyl ester trifluoroacetate[48] (350 mg, 0.9 mmol) in THF (5 mL) and triethylamine (0.13 mL) in THF (5 mL), a solution of HOBT (140 mg, 0.9 mmol) in THF (3 mL), and a solution of DCC (206 mg, 1 mmol) in $CHCl_3$ (3 mL). After being stirred for 1 h at 0 °C, the reaction mixture was stirred overnight at room temperature. The reaction was treated as usual, and a white solid was obtained: yield 710 mg (96%); mp 112–114 °C; TLC $R_f$ (A) 0.35; FAB-MS ($MH^+$) calcd 749, found 749.

***N*-(Benzyloxycarbonyl)-L-tyrosyl-D-threonylglycyl-L-phenylalanyl-L-leucyl-L-serine Benzyl Ester.** To a solution of the preceding compound (710 mg, 0.9 mmol) in MeOH, cooled at 0 °C, was added 1.8 mL of 1 N NaOH. The mixture was stirred at room temperature for 24 h and treated following the standard procedure for alkaline hydrolysis and yielded 442 mg (60%) of a white solid: mp 145–147 °C; TLC $R_f$ (B) 0.82.

To a solution of the preceding compound (395 mg, 0.5 mmol) in THF (5 mL) were added successively a solution of serine benzyl ester hydrochloride (116 mg, 0.5 mmol) and triethylamine (0.07 mL) in $CHCl_3$ (10 mL), a solution of HOBT (77 mg, 0.5 mmol), and a solution of DCC (103 mg, 0.5 mmol). After being stirred for 1 h at 0 °C and 20 h at room temperature, the reaction was treated as usual, and a white solid was obtained: 410 mg (90%); mp 120–122 °C; TLC $R_f$ (A) 0.31; FAB-MS ($MH^+$) calcd 912, found 912.

**L-Tyrosyl-D-threonylglycyl-L-phenylalanyl-L-leucyl-L-serine (20).** A solution of the preceding compound (91 mg, 0.1 mmol) was hydrogenated over Pd/C (10%, 70 mg) in MeOH as described for **15**, yielding 65 mg (95%) of the pure hexapeptide: mp 156–158 °C; TLC $R_f$ (B) 0.32, $R_f$ (C) 0.20; FAB-MS ($MH^+$) calcd 688, found 688. Anal. Calcd for ($C_{33}H_{46}N_6O_{18}$) C, H, N.

***N*-(Benzyloxycarbonyl)-L-tyrosyl-D-threonylglycyl-L-phenylalanyl-L-leucyl-L-leucine Benzyl Ester.** To a solution of *N*-(benzyloxycarbonyl)-L-tyrosyl-D-threonylglycyl-L-phenylalanyl-L-leucine (395 mg, 0.5 mmol) in THF (5 mL) were added successively a solution of leucine benzyl ester *p*-tosylate (197 mg, 0.5 mmol) and triethylamine (0.07 mL) in $CHCl_3$ (10 mL) and a solution of HOBT (77 mg, 0.5 mmol). After being stirred for 1 h at 0 °C and 20 h at room temperature, the reaction was treated as usual, and a white solid was obtained: 398 mg (85%); mp 108–110 °C; TLC $R_f$ (A) 0.52; FAB-MS ($MH^+$) calcd 937, found 937.

**L-Tyrosyl-D-threonylglycyl-L-phenylalanyl-L-leucyl-L-leucine (18).** A solution of the preceding compound was hydrogenated over Pd/C (10%, 70 mg) in MeOH as described for **15**, yielding 59 mg (77%) of the pure hexapeptide: mp 165–167 °C; TLC $R_f$ (B) 0.79, $R_f$ (C) 0.58; FAB-MS ($MH^+$) calcd 713, found 713. Anal. Calcd for ($C_{36}N_{52}N_6O_9$) C, H, N.

**L-Tyrosyl-D-threonylglycyl-L-phenylalanyl-L-leucyl-L-hydroxyproline (21).** To a solution of *N*-(benzyloxy-

carbonyl)-L-tyrosyl-D-threonylglycyl-L-phenylalanyl-L-leucine (395 mg, 0.5 mmol) in THF (5 mL) were added successively a solution of hydroxyproline methyl ester hydrochloride (91 mg, 0.5 mmol) and triethylamine (0.07 mL) in $CHCl_3$ (15 mL), a solution of HOBT (77 mg, 0.5 mmol), and a solution of DCC (113 mg, 0.55 mmol). After 1 h at 0 °C, the reaction mixture was allowed to come to room temperature and stirred overnight. The reaction was treated as usual, and a white solid was obtained: 360 mg (85%); mp 126–128 °C; TLC $R_f$ (A) 0.35.

A solution of the preceding compound (106 mg, 0.13 mmol) was hydrogenated over Pd/C (10%, 70 mg) as described for **15**, yielding 80 mg (86%) of the hexapeptide: mp 172–174 °C; TLC $R_f$ (B) 0.53, $R_f$ (C) 0.45; FAB-MS ($MH^+$) calcd 714, found 714. Anal. Calcd for ($C_{35}H_{48}N_6O_{10}$) C, H, N.

***N*-(Benzyloxycarbonyl)-L-tyrosyl-β-hydroxynorvalyl-glycyl-L-phenylalanyl-L-leucyl-L-threonine Benzyl Ester.** To a solution of *N*-(benzyloxycarbonyl)-L-tyrosine (945 mg, 3 mmol) in THF (15 mL) were added successively a solution of β-hydroxynorvaline methyl ester (550 mg, 3 mmol) and triethylamine (0.42 mL) in $CHCl_3$ (15 mL), a solution of HOBT (460 mg, 3 mmol) in THF (8 mL), and a solution of DCC (618 mg, 3 mmol) in $CHCl_3$ (8 mL). After 1 h at 0 °C, the solution was stirred at room temperature overnight. Classical treatment led to a white solid: 1.23 g (92%); mp 60 °C; TLC $R_f$ (A) 0.40.

To a solution of the preceding compound (889 mg, 2 mmol) in MeOH (8 mL) was added 4 mL of 1 N NaOH at 0 °C, and the reaction mixture was stirred at 0 °C for 1 h and at room temperature for 4 h. The reaction was treated following the standard procedure and produced a white solid: 782 mg (90%); mp 86–88 °C; TLC $R_f$ (C) 0.85. To a sample of the preceding compound (216 mg, 0.5 mmol) dissolved in THF (5 mL) were added successively a solution of glycyl-L-phenylalanyl-L-leucyl-L-threonine benzyl ester (320 mg, 0.5 mmol) and triethylamine (0.07 mL) in $CHCl_3$ (5 mL), a solution of HOBT (77 mg, 0.5 mmol) in THF (3 mL), and a solution of DCC (113 mg, 0.55 mmol) in $CHCl_3$ (3 mL). After 1 h at 0 °C, the reaction mixture was allowed to come to room temperature. Classical treatment yielded a crude product, which was purified by flash chromatography on Kieselgel 60 with $CHCl_3$/MeOH (20:1) as eluent. Fractions containing the pure diprotected hexapeptide were evaporated in vacuo to yield 560 mg (60%): mp 130–132 °C; TLC $R_f$ (A) 0.40; FAB-MS ($MH^+$) calcd 940, found 940.

**L-Tyrosyl-β-hydroxynorvalylglycyl-L-phenylalanyl-L-leucyl-L-threonine (16).** A solution of the preceding compound (60 mg, 0.06 mmol) was hydrogenated over Pd/C (10%, 20 mg) as described for **15**, yielding 40 mg (93%) of the pure hexapeptide: mp 172–174 °C; TLC $R_f$ (B) 0.35, $R_f$ (C) 0.27; FAB-MS ($MH^+$) calcd 716, found 716. Anal. Calcd for ($C_{35}H_{50}N_6O_{11}$) C, H, N.

***N*-(*tert*-Butyloxycarbonyl)-2-azaglycylglycine.** To a solution of *tert*-butyl carbazate (2.640 g, 20 mmol) in $CHCl_3$ cooled in an ice–water bath was added 2.58 g (20 mmol) of ethyl isocyanatoacetate. After 1 h at 0 °C, the reaction mixture was allowed to come to room temperature and stirred for 16 h. The mixture was evaporated in vacuo, and the residue was dissolved in EtOAc (50 mL) and washed successively with $H_2O$ (20 mL), a 10% solution of citric acid (2 × 20 mL), and a saturated solution of NaCl (20 mL). The solvent was dried on $Na_2SO_4$ and evaporated in vacuo: yield 2.96 g (60%); mp 72–74 °C; TLC $R_f$ (A) 0.50.

To a solution of the preceding compound (2.61 g, 10 mmol) in EtOH (10 mL) was added 10 mL of 1 N NaOH at 0 °C, and the reaction mixture was stirred at 0 °C for 1 h and at room temperature for 3 h. The reaction was treated following the standard procedure, and the white solid obtained was recrystallized from a mixture EtOAc/$Et_2O$: yield 1.410 g (60%); mp 140–142 °C; TLC $R_f$ (B) 0.59; FAB-MS ($MH^+$) calcd 234, found 234.

***N*-(*tert*-Butyloxycarbonyl)-L-tyrosyl-2-azaglycylglycyl-L-phenylalanyl-L-leucine Methyl Ester.** To a solution of the preceding compound (1.12 g, 4.8 mmol) in THF (25 mL) were added successively a solution of L-phenylalanyl-L-leucine methyl ester trifluoroacetate (1.95 g, 4.8 mmol) and triethylamine (0.67 mL) in $CHCl_3$ (25 mL), a solution of HOBT (0.65 g, 4.8 mmol), and a solution of DCC (1 g, 4.8 mmol). After 0.5 h at 0 °C, the reaction mixture was stirred at room temperature overnight. Classical treatment yielded 2.2 g (90%) of the pure compound: mp 134 °C; TLC $R_f$ (A) 0.33.

A sample of the preceding compound (0.51 g, 1 mmol) was deprotected as described for **5**, yield 371 mg (71%). To a solution of this compound (365 mg, 0.7 mmol) and triethylamine (0.1 mL) in $CHCl_3$ (5 mL) were added successively, at 0 °C, a solution of *N*-(*tert*-butyloxycarbonyl)-L-tyrosine (221 mg, 0.7 mmol) in THF (5 mL), a solution of HOBT (107 mg, 0.7 mmol), and a solution of DCC (160 mg, 0.77 mmol). After 1 h at 0 °C, the reaction was allowed to come to room temperature and stirred overnight. The reaction was treated as usual, and a white solid was obtained: 460 mg (98%); mp 137–139 °C; TLC $R_f$ (A) 0.5; FAB-MS ($MH^+$) calcd 671, found 671.

***N*-(*tert*-Butyloxycarbonyl)-L-tyrosyl-2-azaglycylglycyl-L-phenylalanyl-L-leucyl-L-threonine Benzyl Ester.** To a solution of the preceding compound (460 mg, 0.69 mmol) in MeOH (10 mL) was added 1.2 mL of 1 N NaOH at 0 °C, and the reaction mixture was stirred at 0 °C for 1 h and at room temperature for 6 h. The reaction was treated following the standard procedure for alkaline hydrolysis and produced a white solid, 415 mg (91%). To a sample of the preceding compound (328 mg, 0.5 mmol) dissolved in THF (5 mL) were added successively a solution of threonine benzyl ester hemioxalate (150 mg, 0.5 mmol) and triethylamine (0.07 mL) in $CHCl_3$ (5 mL), a solution of HOBT (77 mg, 0.5 mmol) in THF (2.5 mL), and a solution of DCC (103 mg, 0.5 mmol) in $CHCl_3$ (2.5 mL). After 1 h at 0 °C and 24 h at room temperature, classical treatment yielded a crude product, which was purified by flash chromatography on Kieselgel 60 with $CHCl_3$/MeOH (20:1) as eluent. Fractions containing the pure deprotected hexapeptide were evaporated in vacuo to yield 264 mg (60%): mp 163–165 °C; TLC $R_f$ (A) 0.43; FAB-MS ($MH^+$) calcd 848, found 848.

**L-Tyrosyl-2-azaglycylglycyl-L-phenylalanyl-L-leucyl-L-threonine (14).** To a solution of the preceding compound (254 mg, 0.33 mmol) in MeOH (10 mL) was added 0.6 mL of 1 N NaOH at 0 °C, and the reaction mixture was stirred at 0 °C for 1 h and at room temperature for 7 h. The reaction was treated following the standard procedure and produced a white solid: 225 mg (89%); mp 168–170 °C; TLC $R_f$ (B) 0.70. A sample of the preceding compound (50 mg, 0.07 mmol) was deprotected as described for **5**. The crude product was purified by silica gel filtration on $LH_{20}$ (Pharmacia) with MeOH as eluent, to yield 40 mg (74%) of the pure hexapeptide: $R_f$ (B) 0.24, $R_f$ (C) 0.16; FAB-MS ($MH^+$) calcd 658, found 658. Anal. Calcd for ($C_{31}H_{43}N_7O_9$): C, H, N.

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