140–143 °C); IR ($CHCl_3$) 3440, 2840–2700 (*trans*-quinolizidine Bohlmann bands) 1435, 1300 cm^{-1}; UV (MeOH) 235, 273, 283, 290 nm; ^1H NMR (250 MHz, $CDCl_3$) δ 7.70 (br s, indole NH), 7.48–7.04 (m, indole H), 5.43 (broadened q, J = 6.8 Hz, C=CH), 3.45–3.3 (m, 2 H), 3.1–2.95 (m, 3 H), 2.85–2.6 (m, 3 H), 2.2–2.1 (m, 1 H), 2.05–1.93 (br t, 1 H), 1.63 (d, J = 6.8 Hz, $C=CCH_3$), 1.65–1.5 (m, 1 H); ^{13}C NMR (63 MHz, $CDCl_3$) δ 136.3, 134.9, 134.3, 127.7, 121.5, 119.6, 119.5, 118.4, 110.9, 108.6, 63.7, 60.4, 53.1, 30.5, 26.1, 21.8, 12.9; mass spectrum (CI, isobutane), m/z (relative intensity, 30% cutoff) 253 (MH^+, 100). Anal. Calcd for $C_{17}H_{20}N_2$: C, 80.91; H, 7.99; N, 11.10. Found: C, 80.89; H, 8.09; N, 11.08.

(*Z*)-3-Ethylidene-1,2,3,4,6,7,12,12b-octahydrolindolo[2,3-*a*]quinolizidine (13). By use of a procedure identical with that described for the preparation of **3**, tetrahydro-β-carboline **9** (542 mg, 1.73 mmol) was cyclized, and the resulting product purified by chromatography and sublimation (140 °C, 0.3 mm) to give 346 mg (79%) of crystalline **13**. Analysis of capillary GC[13] showed that this sample was contaminated with 1.7% of **3** and contained no other detectable impurities. An analytical sample of **13** was prepared by one additional sublimation (140 °C, 0.3 mm): mp 163 °C (lit.[3a] 148–153 °C); IR ($CHCl_3$) 3480, 2930, 2850–2745 (*trans*-quinolizidine Bohlmann bands), 1450, 1320 cm^{-1}; UV (MeOH) 235, 274, 282, 289 nm; ^1H NMR (250 MHz, $CDCl_3$) δ 7.70 (br s, indole NH), 7.5–7.05 (m, indole H), 5.33 (broadened q, J = 6.8 Hz, C=CH), 3.81 (br d, J = 12.3 Hz, C_{21} H) 3.43, (br dd, J = 1.7, 11.2 Hz, 1 H), 3.2–2.95 (m, 2 H), 2.8–2.65, (m, 3 H), 2.45–2.3 (m, 2 H), 2.2–2.05 (m, 1 H), 1.7–1.6 (m, 1 H), 1.66 (d, J = 6.9 Hz, $C=CCH_3$); ^{13}C NMR (63 MHz, $CDCl_3$) δ 136.2, 135,0, 134.0, 127.5, 121.3, 119.3, 118.8, 118.2, 110.9, 108.1, 60.0, 55.1, 53.1, 34.1, 30.9, 21.7, 12.9; mass spectrum (CI, isobutane), m/z (relative intensity, 35% cutoff) 253 (MH^+, 100), 252 (42). Anal. Calcd for $C_{21}H_{24}N_2O_4$ (maleate salt): C, 68.46; H, 6.57; N, 7.60. Found: C, 68.25; H, 6.70; N, 7.46.

Acknowledgment. Financial assistant from the National Institutes of Health (Grant HL25854) and the Camille and Henry Dreyfus Foundation (Teacher-Scholar award to L.E.O.) is gratefully acknowledged. NMR and mass spectra were determined with spectrometers purchased with the assistance of NSF departmental instrumentation grants. We particularly thank Dr. H.-P. Husson for copies of spectra and Professor J. A. Joule for authentic samples of *dl*-deplancheine and a mixture of *dl*-deplancheine and **13**.

Registry No. (±)-**3**, 56491-03-3; **4**, 6224-91-5; **5**, 83665-51-4; **6**, 83665-52-5; **7**, 83665-56-9; **8**, 83681-25-8; (±)-**9**, 83665-53-6; (±)-**10**, 83665-54-7; (±)-**11**, 65601-17-4; (±)-**12**, 65601-15-2; (±)-**13**, 76549-66-1; (±)-**13** maleate, 83665-57-0; 2-(2-iodoethyl)-1,3-dioxolane, 83665-55-8.

Enzymatic Synthesis of Dynorphin (1–8)

Willi Kullmann

Max-Planck-Institut für Biophysikalische Chemie, D-3400 Göttingen, Am Fassberg, Federal Republic of Germany

Received August 12, 1982

Conventional syntheses of arginyl peptides are often accompanied by undesired side reactions. An alternative approach is proposed to overcome these difficulties which exploits the proteosynthetic potential of trypsin. The enzyme's specificity ensures regio- and stereocontrolled synthesis of peptide bonds whose carbonyl moiety is associated with a basic amino acid residue. To demonstrate the feasibility of the enzymatic method all the peptide bonds of a biologically active dynorphin (1–8) (primary structure H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-OH) were prepared by protease catalysis as follows: the protected octapeptide **31** was obtained via α-chymotrypsin-mediated condensation of Boc-Tyr(Bzl)-Gly-Gly-Phe-OEt (**13**) and H-Leu-Arg-Arg-Ile-N_2H_2Ph. The preparation of **13** has already been described.[15] Boc-Leu-Arg-Arg-Ile-N_2H_2Ph (**30**) was synthesized from Boc-Leu-Arg-OMe (**28**) and H-Arg-Ile-N_2H_2Ph in the presence of trypsin. Boc-Leu-Arg-N_2H_2Ph (**27**) the precursor of **28**, was prepared by α-chymotrypsin-catalyzed coupling of Boc-Leu-OEt and H-Arg-N_2H_2Ph. Boc-Arg-Ile-N_2H_2Ph (**29**) was obtained by tryptic condensation of Boc-Arg-OMe and H-Ile-N_2H_2Ph.

Although the recent development of new methods for the chemical coupling of appropriate protected amino acid or peptide derivatives has improved significantly the prospects of peptide synthesis, the preparation of arginine peptides still represents an intricate procedure.[1-3] Despite the strongly basic character of the δ-guanidine group of arginine (pK_a = 12.5), which is generally protonated under conditions prevailing in peptide synthesis, the low solubility in organic solvents of charged arginine derivatives and their tendency to form lactams during the activation of the carboxyl group often necessitates N^G protection. However, the nucleophilicity of the δ-guanidine group, even in the protected form, is high enough for an intramolecular reaction of the carbonyl with the vicinal guanidine nitrogen, resulting in cyclization to piperidinones.[2] According to Bodanszky and Martinek,[2] complete protection to fully suppress the basicity of the δ-guanidine group requires N^δ, N^ω-diacylation by bulky protecting groups. On the other hand, as stated by Barany and Merrifield,[4] the advantages of this kind of protection are counterbalanced by considerations of ease of synthesis, potential steric hindrances during coupling, and selective removal of N^α-protecting groups. Therefore, biologically active oligopeptides containing arginine residues or even Arg-Arg subsequences are difficult to prepare. In this paper I propose an alternative approach to the synthesis of arginyl peptides which relies on the capacity of trypsin to catalyze peptide bond formation when the amino acid which contributes the carbonyl portion of the bond to be formed is an arginine or a lysine residue. The regio- and stereospecificity of tryptic action prevents the occurrence of undesired side reactions

(1) Wünsch, E. In "Methoden der Organischen Chemie (Houben-Weyl)"; Müller, E., Ed.; Georg Thieme Verlag: Stuttgart, 1974; Vol. 15, Part I.
(2) Bodanszky, M.; Martinek, J. *Synthesis* **1981**, 333.
(3) Geiger, R.; König, W. In "The Peptides"; Gross, E., Meienhofer, J., Eds.; Academic Press: New York, 1981; Vol. 3, pp 1–99.
(4) Barany, G.; Merrifield, R. B. In "The Peptides"; Gross, E., Meienhofer, J., Eds.; Academic Press: New York, 1980; Vol. 2, pp 1–284.

0022-3263/82/1947-5300$01.25/0

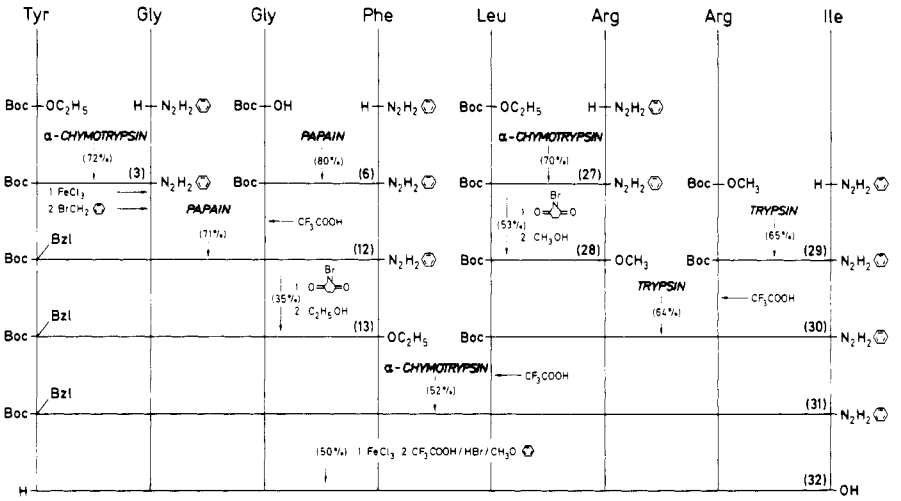

Figure 1. Protease-catalyzed synthesis of dynorphin (1–8).

such as lactam formation and racemization which is a problem of major concern in peptide synthesis.

The first report on a trypsin-catalyzed synthesis of a well-defined peptide bond was published by Sealock and Laskowski, Jr.,[5] in 1969. This study on the replacement of an arginine by a lysine residue in soybean trypsin inhibitor suggested the promising features of trypsin in enzymatic peptide bond formation. Since then the proteosynthetic capacity of trypsin has been exploited mainly for the "semisynthesis" of enzymes, protein hormones, and protease inhibitors (for reviews, see ref 6–11). Although trypsin has also been used during several systematic studies dealing with the preparation of model peptides,[12–14] the protease has heretofore not yet been applied to the synthesis of biologically active peptides.

Enzymatic syntheses of the enkephalins in which all the peptide bonds were prepared by protease-catalyzed peptide bond formation have recently been described.[15,16] On the basis of my previous studies on the synthesis of Leu-enkephalin,[15] the sequence of which constitutes the N-terminal pentapeptide portion of dynorphin, I wished to explore further the potential of enzymatic peptide synthesis, placing particular emphasis on the capability of trypsin to catalyze the synthesis of naturally occurring arginyl peptides. It was therefore decided to prepare enzymatically all the peptide bonds of the subsequence (1–8) of dynorphin

H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-OH

by using a combined action of papain, α-chymotrypsin, and trypsin catalysis.

The occurrence of this opioid octapeptide in the porcine hypothalamus and the pituitary of rats has recently been reported.[17,18] Its amino acid sequence is identical with the N-terminal portion (1–8) of dynorphin, the isolation of which was described by Goldstein et al.[19] The complete sequence of the heptadecapeptide was determined.[20,21]

The synthetic pathway leading to the dynorphin-related octapeptide is illustrated in Figure 1.

Throughout this work, α-carboxyl protection of N^{α}-Boc amino acids and peptides was achieved by phenylhydrazide groups which were conveniently introduced by papain catalysis.[22] During previous studies on enzymatic synthesis[15,23] the above blocking groups were found to be easily and selectively removable. Boc amino acid or peptide esters served as carboxyl components during trypsin and α-chymotrypsin catalysis. As can be deduced from the results in the current literature,[12–14,24,25] ester donors are markedly more efficient for both trypsin- and α-chymotrypsin-catalyzed synthesis than acyl group donors having a free carboxyl group. The enzymatic reactions took place at pH 10, where the proteolytic activity is low and the esterase potential high. Consequently, the risk of a posteriori hydrolysis which may frustrate an otherwise successful synthesis, as has happened with the trypsin-catalyzed resynthesis of nuclease-T fragments,[26] is largely reduced.

Boc-Leu-Arg-N_2H_2Ph (**27**) was synthesized in 70% yield via α-chymotrypsin catalysis from Boc-Leu-OEt and H-Arg-N_2H_2Ph. The reaction required a long incubation period (1 h) relative to those reported by Morihara and Oka.[24] This finding corresponds to the results of Kloss and Schröder,[27] who found that leucine esters were poorer substrates for α-chymotrypsin-catalyzed esterolysis than esters of aromatic amino acid residues. The prospective donor ester Boc-Leu-Arg-OMe (**28**) was obtained in 53%

(5) Sealock, R. W.; Laskowski, M., Jr. *Biochemistry* **1969**, *8*, 3703.
(6) Laskowski, M., Jr. In "Semisynthetic Peptides and Proteins"; Offord, R. E., Di Bello, C., Eds.; Academic Press: New York, 1978; pp 256–262.
(7) Kowalski, D. In "Semisynthetic Peptides and Proteins"; Offord, R. E., Di Bello, C., Eds.; Academic Press: New York, 1978; pp 263–282.
(8) Jering, H.; Tschesche, H. In "Semisynthetic Peptides and Proteins"; Offord, R. E., Di Bello, D., Eds.; Academic Press: New York, 1978; pp 283–298.
(9) Chaiken, I. M. *CRC Crit. Rev. Biochem.* **1981**, *11*, 255.
(10) Fruton, J. S. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1982**, *53*, 239–306.
(11) Jakubke, H.-D.; Kuhl, P. *Pharmazie* **1982**, *37*, 89.
(12) Oka, T.; Morihara, K. *J. Biochem.* **1977**, *82*, 1055.
(13) Inouye, K.; Watanabe, K.; Morihara, K.; Tochino, T.; Kanaya, T.; Emura, J.; Sakakibara, S. *J. Am. Chem. Soc.* **1979**, *101*, 751.
(14) Tsuzuki, H.; Oka, T.; Morihara, K. *J. Biochem.* **1980**, *88*, 669.
(15) Kullmann, W. *J. Biol. Chem.* **1980**, *255*, 8234.
(16) Widmer, F.; Breddam, K.; Johansen, J. T. In "Proceedings of the 16th European Peptide Symposium"; Brunfeldt, K., Ed.; Scriptor: Copenhagen, 1981, pp 46–55.
(17) Minamino, N.; Kangawa, K.; Fukuda, A.; Matsuo, H.; Iagarashi, M. *Biochem. Biophys. Res. Commun.* **1980**, *95*, 1475.
(18) Seizinger, B. R.; Hoellt, V.; Herz, A. *Biochem. Biophys. Res. Commun.* **1981**, *102*, 197.
(19) Goldstein, A.; Tachibana, S.; Lowney, L. I.; Hunkapiller, M.; Hood, L. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 6666.
(20) Goldstein, A.; Fischli, W.; Lowney, L. I.; Hunkapiller, M.; Hood, L. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 7219.
(21) Tachibana, S.; Anaki, K.; Ohya, S.; Yoshida, S. *Nature (London)* **1982**, *295*, 339.
(22) Milne, H. B.; Carpenter, F. H. *J. Org. Chem.* **1968**, *33*, 4476.
(23) Kullmann, W. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 2840.
(24) Morihara, K.; Oka, T. *Biochem. J.* **1977**, *163*, 531.
(25) Oka, T.; Morihara, K. *J. Biochem. (Tokyo)* **1978**, *84*, 1277.
(26) Homandberg, G. A.; Chaiken, I. M. *J. Biol. Chem.* **1980**, *255*, 4903.
(27) Kloss, G.; Schröder, E. *Hoppe-Seyler's Z. Physiol. Chem.* **1964**, *336*, 248.

yield by a method analogous to that of Milne and Kilday,[28] involving the successive treatment of Boc-Leu-Arg-N_2H_2Ph (**27**) with *N*-bromosuccinimide and methanol. Boc-Arg-Ile-N_2H_2Ph (**29**), the acylated form of the prospective acceptor nucleophile, was easily prepared by allowing Boc-Arg-OMe to react with H-Ile-N_2H_2Ph in the presence of trypsin (yield, 65%).

Boc-Leu-Arg-Arg-Ile-N_2H_2Ph (**30**) was prepared in 64% yield by the trypsin-mediated condensation of Boc-Leu-Arg-OMe (**28**) and H-Arg-Ile-N_2H_2Ph. The trypsin-sensitive Arg–Ile bond was scarcely affected during this reaction probably due to the relatively high pH (10.4) of the reaction medium, at which tryptic hydrolysis is largely suppressed. This observation agrees with the results of Oka and Morihara,[12] who succeeded in synthesizing Bz-Arg-Lys-Leu-OH from Bz-Arg-OEt and H-Lys-Leu-OH. Furthermore, tryptic treatment of dynorphin (1–8) did not result in cleavage of the Arg–Ile bond, as reported by Minamino et al.[17]

The dynorphin-related octapeptide Boc-Tyr(Bzl)-Gly-Gly-Phe-Leu-Arg-Arg-Ile-N_2H_2Ph (**31**) was obtained in 52% yield by α-chymotrypsin-controlled coupling of Boc-Tyr(Bzl)-Gly-Gly-Phe-OEt (**13**)[29] and H-Leu-Arg-Arg-Ile-N_2H_2Ph, the deacylated form of **30**. Although the Leu–Arg bond cannot be considered to be completely inert toward α-chymotrypsin action (see above), degradation products were not traceable. This appears reasonable in the view of the reaction conditions (pH 10.1), which reduce the enzyme's peptidase activity. It is also consistent with the observation of Kangawa et al.[30] that α-chymotrypsin cleaved primarily the Phe–Leu linkage and the Leu–Arg bond to a lesser extent. As compared to the reaction conditions previously described by Morihara and Oka,[24] the preparation of **31** required a prolonged incubation period, an elevated temperature (38 °C), and an increased enzyme concentration (4×10^{-4} M). Under these circumstances it took 12 h until the donor ester **13** had been completely utilized. A similar behavior of **13** which is probably based on an inhibitiory effect contributed by the benzylated tryosine residue has been observed and discussed during the study on the synthesis of Leu-enkephalin.[15]

Trypsin-[31] and α-chymotrypsin[32] -catalyzed reactions are believed to proceed via an acyl–enzyme complex. In the subsequent deacylation process, preferential peptide bond formation takes place if aminolytic cleavage of the complex successfully competes with hydrolysis. The degree of peptide synthesis thus depends strongly on the nucleoplicity and the concentration of the amine component. Furthermore, a high concentration of amine component inhibits the secondary hydrolysis of the newly formed peptide bonds, as far as α-chymotrypsin is concerned.[33] Analogously, this may also hold true in the case of trypsin.[12] For shifting of the trypsin- and α-chymotrypsin-mediated reactions in favor of peptide synthesis at the expense of esterolysis and for suppression of secondary hydrolysis, the concentration of the amine components was increased twofold relative to the amount of donor ester. By doubling the amine concentration, one could improve the product yield as follows: **27**, 40%; **29**, 18%; **30**, 22%; **31**, 15%. The enzymatic reactions were allowed to proceed until the respective donor ester was completely exhausted. During this period there was no evidence that secondary hydrolysis had occurred. The final ratio of yields of peptide synthesis to ester hydrolysis was found to be (approximately) as follows: **27**, 9:1; **29**, 8:2; **30**, 8:2; **31**, 7:3.

The protected dynorphin octapeptide **31** was treated with $FeCl_3$ and then with HBr/TFA to release the free octapeptide **32**. After purification via gel filtration, ion-exchange partition chromatography, and reversed-phase HPLC, **32** was chromatographically and electrophoretically homogeneous and comigrated with chemically prepared dynorphin (1–8) on thin-layer chromatograms and electropherograms (yield, 50%). The synthetic dynorphin (1–8) (**32**) was 11 times more potent than Leu-enkephalin and 16 times more potent than Met-enkephalin in the opiate receptor binding assay.

Leu-enkephalin, the primary structure of which is identical with that of dynorphin (1–5), was recovered from dynorphin (1–8) (**32**) by successive removal of the three COOH terminal amino acid residues. Ile_8, Arg_7, and Arg_6 were quantitatively released in this order upon consecutive treatment of **32** with CPase-A, trypsin, and CPase-B. The dynorphin (1–5) fragment was chemically and biologically (opiate receptor assay) indistinguishable from enzymatically prepared Leu-enkephalin.[15]

In an extension of the synthesis of Leu-enkephalin[15] all the peptide bonds of the "big"-enkephalin[17,18] dynorphin (1–8) containing the critical Arg-Arg sequence were prepared by protease-mediated synthesis. As a consequence of the stereo- and regioselective action of the proteases, the enzymatic approach opens up a convenient way to prepare, under mild conditions, complex peptides whose synthesis is often accompanied by sequence-dependent side reactions.

Experimental Section

tert-Butyloxycarbonyl (Boc) amino acids were purchased from Bachem and were of L configuration. Trypsin (EC 3.4.21.4; diphenylcarbamoyl treated), α-chymotrypsin (EC 3.4.21.1; tosyllysylchloromethane treated), CPase-A (EC 3.4.17.1; diisopropyl fluorophosphate treated), and CPase-B (EC 3.4.17.2; diisopropyl fluorophosphate treated) were obtained from Sigma. Boc amino acid methyl or ethyl esters and phenylhydrazides were prepared as described.[15] Removal of the phenylhydrazide with $FeCl_3$ and of *tert*-butyloxycarbonyl groups with CF_3COOH was accomplished as reported.[15] After CF_3COOH treatment, the deacylated amino acids or peptide trifluoroacetates were dissolved in methanol–water (1:1) and stirred with Amberlite IRA (Serva), following which the ion-exchange resin was removed by filtration, and the filtrate was evaporated under reduced pressure. Chemically prepared dynorphin (1–8) was obtained from the Peptide Institute, Inc. (Osaka, Japan). Prepacked LiChroprep Si 60 (310 × 25 mm; 40–63 μm) and LiChrosorb RP-18 (250 × 4 mm; 10 μm) columns for HPLC (high-performance liquid chromatography) were purchased from Merck. HPTLC (high-performance thin-layer chromatography) was performed on precoated silica gel plates (Merck) by using the following solvent systems: A, chloroform/methanol (3:1); B, chloroform/methanol/acetic acid (9:3:1); C, 1-butanol/pyridine/acetic acid/water (5:5:1:3). Amino acid hydrolyses (6 M HCl) were carried out in sealed tubes at 110 °C for 24 h under reduced pressure. Enzymatic hydrolyses were performed as follows: the respective dynorphin fragments were incubated with either CPase-A, trypsin, or CPase-B [peptide–enzyme ratio of 4:1 (w/w) in 0.1 M Tris buffer (pH 8.0) for 40 min (trypsin 6 h) at 37 °C]. Quantitative determination of the amino acid residues released was carried out on an amino acid analyzer.

Boc-Leu-Arg-N_2H_2Ph (27). Boc-Leu-OEt (777 mg, 3 mmol) and H-Arg-N_2H_2Ph (1.58 g, 6 mmol) dissolved in 30 mL of 0.2 M carbonate buffer/dimethylformamide (9:1; pH 10.1) were incubated in the presence of α-chymotrypsin (136 mg) under vigorous stirring. The reaction proceeded for 1 h at room temperature

(28) Milne, H. B.; Kilday, W. *J. Org. Chem.* **1965**, *30*, 64.
(29) A detailed description of the synthesis of **13**, which also served as donor ester during the preparation of Leu-enkephalin, has been given elsewhere.[15]
(30) Kangawa, K.; Minamino, N.; Chino, N.; Sakakibara, S.; Matsuo, H. *Biochem. Biophys. Res. Commun.* **1981**, *99*, 871.
(31) Keil, B. In "The Enzymes"; Boyer, P. D., Ed.; Academic Press: New York, 1971; Vol. III, pp 249–275.
(32) Blow, D. M. *Acc. Chem. Res.* **1976**, *9*, 145.
(33) Fastrez, J.; Fersht, A. R. *Biochemistry* **1973**, *12*, 2025.

and was then stopped by acidification to pH 3.1 by 1 N HCl. The reaction mixture was evaporated to dryness under reduced pressure (<10^{-3} psi, 30 °C). The residue was taken up in a small volume of dimethylformamide (insoluble material was removed by filtration) and fractionated by preparative HPLC on a prepacked silica gel 60 column. Elution with chloroform/methanol (20:1) provided the pure dipeptide **27** which was crystallized from ethyl acetate/ether; yield 1.00 g (2.1 mmol, 70% based on Boc-Leu-OEt). HPTLC analysis of **27** showed a single spot at R_f 0.29 (system A) and at R_f 0.64 (system B): HPLC analysis on LiChrosorb RP-18 [eluant, 0.05 M KH_2PO_4/CH_3OH (2:3); flow-rate, 1.5 mL/min] $k' = 2.2$;[34] mp 145–148 °C; $[\alpha]^{22}_D$ −10.1° (*c* 0.7, DMF). Anal. Calcd for $C_{23}H_{39}N_7O_4$: C, 57.84; H, 8.23; N, 20.53. Found: C, 57.76; H, 8.13; N, 20.31.

Boc-Leu-Arg-OMe (28). Boc-Leu-Arg-N_2H_2Ph (**27**; 764 mg, 1.60 mmol) was oxidized with *N*-bromosuccinimide (312 mg, 1.75 mmol), and the resulting dipeptide diimide was then treated with methanol (6 mL). The preparation of **28** and the workup conditions followed those described for Boc-Gly-Phe-OEt.[15] The pure dipeptide **28** was obtained via HPLC on a silica gel 60 column [chloroform/methanol (30:1) eluant]. Crystallization from acetone/ether yielded the product: 340 mg (0.85 mmol, 53%); HPTLC r_f 0.43 (system A), 0.74 (system B); HPLC [RP-18; eluant, 0.05 M KH_2PO_4/CH_3OH (2:3); flow-rate, 1.5 mL/min] $k' = 3.4$;[34] mp 89–91 °C; $[\alpha]^{22}_D$ −15.3° (*c* 1, DMF). Anal. Calcd for $C_{18}H_{35}N_5O_5$: C, 53.85; H, 8.79; N, 17.45. Found: C, 54.06; H, 9.01; N, 17.17.

Boc-Arg-Ile-N_2H_2Ph (29). A solution of Boc-Arg-OMe (1.15 g, 4 mmol) and H-Ile-N_2H_2Ph (1.77 g, 8 mmol) in 20 mL of 0.5 M carbonate buffer (pH 10.5) was incubated with trypsin (72 mg) under vigorous stirring at room temperature. The reaction was terminated after 10 min by acidification to pH 3.4 with 1 N HCl. The workup conditions were those described for compound **27**. Pure **29** was eluted from a prepacked silica gel 60 column with chloroform/methanol (25:1) and was crystallized from ethyl acetate/ether to give **29**: 1.24 g (2.6 mmol, 65% based on Boc-Arg-OMe); HPTLC R_f 0.31 (system A), 0.69 (system B); HPLC [RP-18; eluant, 0.05 M KH_2PO_4/CH_3OH (2:3); flow-rate, 1.5 mL/min] $k' = 4.1$; mp 130–132 °C; $[\alpha]^{22}_D$ −7.8° (*c* 1, DMF). Anal. Calcd for $C_{23}H_{39}N_7O_4$: C, 57.84; H, 8.23; N, 20.53. Found: C, 58.14; H, 8.25; N, 20.45.

Boc-Leu-Arg-Arg-Ile-N_2H_2Ph (30). Trypsin (13 mg) was added under vigorous stirring to a solution of Boc-Leu-Arg-OMe (**28**; 292 mg, 0.72 mmol) and H-Arg-Ile-N_2H_2Ph (543 mg, 1.44 mmol) in 3.6 mL of 0.5 M carbonate buffer (pH 10.5) at room temperature. After 5 min, the reaction was terminated by acidification (pH 3.2) with 1 N HCl. The reaction mixture was worked up as described for **27**. Preparative HPLC on a silica gel 60 column gave the pure tetrapeptide **30** [chloroform/methanol/acetic acid (30:1:1) eluant]. Crystallization from ethanol/water provided **30**: 347 mg (0.46 mmol, 64% based on **28**); HPTLC R_f 0.45 (system A), 0.72 (system B); HPLC [RP-18; eluant, 0.05 M KH_2PO_4/CH_3OH (3:7); flow-rate, 1.5 mL/min] $k' = 1.5$; mp 138–141 °C dec; $[\alpha]^{22}_D$ −14.1° (*c* 1.5, DMF). Anal. Calcd for $C_{35}H_{62}N_{12}O_6$: C, 56.29; H, 8.37; N, 22.50. Found: C, 56.19; H, 8.00; N, 22.71. Amino acid analysis: Ile, 0.94 (1); Leu, 1.00 (1); Arg, 1.92 (2).

Boc-Tyr(Bzl)-Gly-Gly-Phe-Leu-Arg-Arg-Ile-N_2H_2Ph (31). A suspension of Boc-Tyr(Bzl)-Gly-Gly-Phe-OEt (**13**;[15] 132 mg, 0.2 mmol) and H-Leu-Arg-Arg-Ile-N_2H_2Ph (258 mg, 0.4 mmol) in 2 mL of 0.2 M carbonate buffer/dimethylformamide (3:2; pH 10.1) was incubated under vigorous stirring with α-chymotrypsin (18 mg) at 37 °C. The reaction proceeded for 12 h and was then stopped by acidification to pH 3.2 by 1 N HCl. The workup conditions were those described for **27**. The pure octapeptide **31** was eluted from a silica gel 60 column with chloroform/methanol (30:1) and was then crystallized from ethyl acetate–ether to provide **31**: 131 mg (0.104 mmol, 52% based on **13**); HPTLC R_f 0.54 (system A), 0.86 (system B); HPLC [RP-18; eluant, 0.05 M KH_2PO_4/CH_3OH (3:7); flow-rate, 1.5 mL/min] $k' = 10.7$; mp 117–120 °C; $[\alpha]^{22}_D$ −6.5° (*c* 0.8 in DMF). Anal. Calcd for $C_{64}H_{92}N_{16}O_{11}$: C, 60.94; H, 7.35; N, 17.77. Found: C, 61.23; H, 7.04; N, 17.63. Amino acid analysis; Gly, 2.00 (2); Ile, 0.89 (1); Leu, 1.02 (1); Tyr, 0.92 (1); Phe, 1.04 (1), Arg, 1.87 (2).

H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-OH (32). A solution of $FeCl_3 \cdot 6H_2O$ (135 mg, 0.5 mmol) in 2 mL of water was slowly added to a suspension of **31** (100 mg, 0.08 mmol) in 3 mL of dioxane. The suspended material was dissolved on stirring at 36 °C, and the reaction proceeded until nitrogen evolution ceased. The workup conditions followed those described for compound XVI in ref 15. The residual oil was dissolved in 8 mL of CF_3COOH containing 0.4 mL of anisole, and a slow stream of anhydrous HBr was bubbled through the solution. After 60 min, the solvents were evaporated under reduced pressure, and the remaining free octapeptide **32** was precipitated with ether. The precipitate was filtered off, dried in vacuo over NaOH, and fractionated on a Bio-Gel P-2 column with 0.05 M NH_4HCO_3 as the eluant. Further purification was achieved via (a) ion-exchange chromatography on a CM-cellulose column, eluting with a linear gradient from 0.01 to 0.1 M CH_3COONH_4, (b) partition chromatography on a silica gel 60 column, eluting with the organic layer of the system 1-butanol/acetic acid/water (4:1:5), and (c) reversed-phase HPLC on a prepacked LiChrosorb RP-18 column with 0.05 M KH_2PO_4/CH_3OH (1:1) as the eluant; yield 39 mg (0.04 mmol 50%). The synthetic octapeptide **32** was chromatographically and electrophoretically homogeneous: HPTLC R_f 0.63 (system C), 0.83 (system D); HPLC [RP-18; eluant, 0.05 M KH_2PO_4/CH_3OH (1:1); flow-rate, 1.5 mL/min] $k' = 2.1$. On thin-layer electrophoresis in 0.1 M pyridinium acetate buffer (pH 5.8, 400 V, 30 min) on a precoated cellulose plate the synthetic material moved as a single component and had a smaller mobility (5 cm) toward the cathode than H-Arg-OH (9 cm); $[\alpha]^{22}_D$ −14.8° (*c* 0.7, H_2O). Amino acid analysis: Gly, 2.00 (2); Ile, 0.95 (1); Leu, 0.97 (1); Tyr, 0.93 (1); Phe, 1.02 (1); Arg, 1.98 (2).

In opiate receptor binding assays according to Bedell et al.[35] synthetic dynorphin (1–8) was shown to compete with [^{3}H]naloxone (1 nM) for specific binding[36] to whole rat brain homogenates. The potency of **32**, expressed as the concentration which causes 50% inhibition of the radioligand binding (IC_{50}), was compared with the respective potencies of some enkephalin derivatives. IC_{50}: dynorphin (1–8) (**32**); 1.2×10^{-9} M; Met-enkephalin,[15] 1.9×10^{-8} M; Leu-enkephalin,[15] 1.3×10^{-8} M; *all*-D-Leu-enkephalin,[37] $>10^{-6}$ M.

Registry No. **13**, 75488-73-2; **27**, 83731-83-3; **28**, 83731-84-4; **29**, 83731-85-5; **30**, 83731-86-6; **31**, 83731-87-7; **32**, 75790-53-3; α-chymotrypsin, 9004-07-3; trypsin, 9002-07-7; dynorphin, 74913-18-1; Boc-Leu-OEt, 37787-76-1; H-Arg-N_2H_2Ph, 83731-78-6; Boc-Arg-OMe, 83731-79-7; H-Ile-N_2H_2Ph, 83731-80-0; H-Arg-Ile-N_2H_2Ph, 83731-81-1; H-Leu-Arg-Arg-Ile-N_2H_2Ph, 83731-82-2.

(34) The capacity factor k' was determined as follows: $k' = (V_R - V_0)/V_0$, where V_R is the retention volume of the respective peptide and V_0 is the dead volume.

(35) Bedell, C. R.; Clark, R. B.; Hardy, G. W.; Lowe, L. A.; Ubatuba, F. B.; Vane, J. R.; Wilkinson, S.; Chang, K.-J.; Cuatrecasas, P.; Miller, R. J. *Proc. R. Soc. London, Ser. B* **1977**, *198*, 249.

(36) Displacement of the radioligand (1 nM [^{3}H]naloxone) by a large excess of enzymatically prepared Leu-enkephalin (1 μM) was taken as a measure for specific receptor binding.

(37) Kullmann, W. *Biochem. Biophys. Res. Commun.* **1979**, *91*, 693.