Biodegradable Microspheres. 16. Synthesis of Primaquine–Peptide Spacers for Lysosomal Release from Starch Microparticles

ROSSITSA BORISSOVA*‡, BERNARD LAMMEK§, PETER STJÄRNKVIST*‡||, AND INGVAR SJÖHOLM*x

Received June 1, 1993, from the *Division of Pharmacy, Medical Products Agency, Box 26, S-751 03 Uppsala, Sweden, ‡Department of Pharmaceutical Biosciences, University of Uppsala, Box 578, S-751 23 Uppsala, Sweden, §Department of Chemistry, University of Gdansk, 80-952 Gdansk, Poland. Accepted for publication August 17, 1994⊗ ||Present address: Innovex Nordic AB, Dragarbrunnsgatan 31, S-753 20 Uppsala, Sweden.

Abstract □ Classical procedures of peptide synthesis were applied to synthesize four groups of compounds, and analytical methods were developed for each of them. Two of the groups are tetrapeptide derivatives of the antileishmanial drug primaquine (PQ), with general structure NH_2-X-Leu-Ala-Y-PQ. In the first group, Leu, Tyr, Lys, and Asp were used in the Y position, while X was Ala. In the second group, Ala, Tyr, Lys, and Asp were used in the X position, while Y was Leu. The derivatives are intended to be coupled, via their free α-amino group, to polyacryl starch microparticles, lysosomotropic drug carriers developed in our laboratory. Thus, a systematic study of the significance of the varying amino acid composition of the tetrapeptide spacer arm for the rate of lysosomal enzymatic release of PQ can be possible. A third group, comprising ϵ-aminocaproic acid–PQ derivatives which lack a free α-amino group, was synthesized. This was done to study the importance of enzymes, other than aminopeptidases, during lysosomal degradation of these derivatives. To allow HPLC analysis of the pattern of degradation of tetrapeptide–PQ derivatives, some shorter peptide–PQ derivatives (group four) were prepared as well.

Introduction

A possible approach to treat lysosomal parasitic diseases, e.g. leishmaniasis, is targeting of drugs to the infected lysosomes via lysosomotropic drug carriers.[1] Polyacryl starch microparticles (Mp) have been shown to target drugs to the reticuloendothelial system (RES), mainly the lysosomes of the liver macrophages,[2] where *Leishmania donovani* parasites are localized. Proteins can be physically entrapped within the microparticles during their production[3] or covalently bound directly to the starch matrix with e.g. carbonyldiimidazole.[4] Low molecular drugs, however, must be covalently bound to the particles via peptide spacer arms.[5] Within the lysosomes, such spacer arms are cleaved by hydrolytic enzymes and the drug subsequently released in an active form.[5,6]

We have earlier shown that the activity of the drug primaquine (PQ) is significantly improved in the treatment of mice visceral leishmaniasis when the drug is bound to the starch microparticles via an Ala-Leu-Ala-Leu spacer arm.[7]

The pharmacological activity and pharmacokinetics of any microparticle–peptide–drug conjugate used *in vivo* will depend on the cleavage of the polypeptide spacer arm and the subsequent release of the free drug. The lysosomal vacuome of the Kupffer cells is known to contain about 50 hydrolases,[8] among them both endo- and exopeptidases, which together can cleave the spacer arm and release the free drug from the carrier. However, the specificity and relative activity of the different enzymes participating in the release of the active drug or the structure of their active sites are not yet known.[9]

The present work describes the synthesis of many new peptide–PQ derivatives as well as several peptide intermediates. Some of the former were incubated with rat liver lysosomal fractions in order to study *in vitro* the pattern and rate of release of PQ by rat liver lysosomal enzymes, the results of which are presented in the paper immediately following this one.[10]

In order to elucidate the specificity and activity of the lysosomal enzymes, four groups of peptide–PQ derivatives were synthesized. In the first one (Scheme 1, compounds **8**, **12**, **16**, and **20**), the C-terminal amino acid Y in NH_2-X-Leu-Ala-Y-PQ was varied, being Leu, Tyr, Lys, or Asp, while X was in all cases Ala. In the second one (Scheme 2, compounds **28**, **32**, **36**, and **40**), the N-terminal amino acid X was similarly varied, being Ala, Tyr, Lys, or Asp, while Y was in all cases Leu. Thereby, the effects of neutral, aromatic, basic, and acidic amino acids on the pattern and rate of release of PQ by the lysosomal enzymes could be systematically studied.

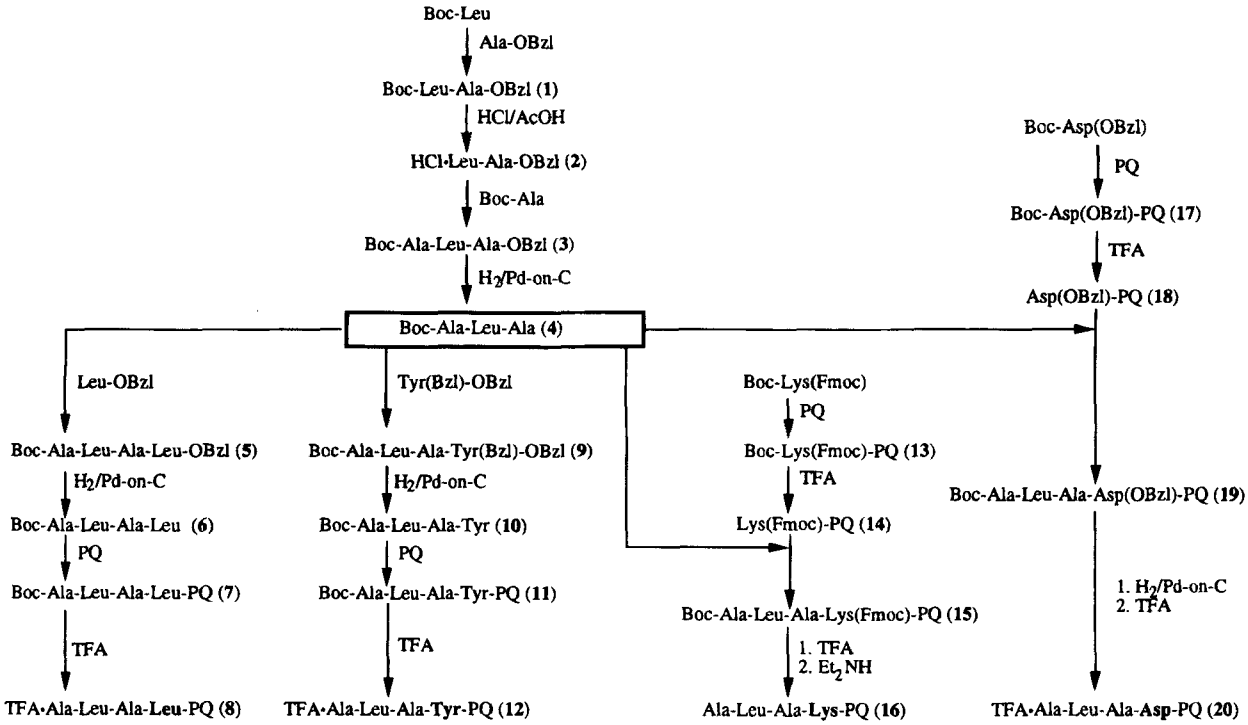
In a third series of PQ–peptides (Scheme 3, compounds **42**, **44**, and **48**) an ϵ-aminocaproic acid (EACA) was introduced as the N-terminal amino acid. EACA cannot be released from such a position by any known aminopeptidase, as an α-amino group is missing. These derivatives can therefore be used to study the presence of any enzymes releasing PQ directly from the C-terminus of the derivatives and the specificity of any endopeptidase activity in the lysosomes. Finally, Scheme 4 (compounds **49**–**53** and **55**–**59**) shows the synthesis of some tripeptide–, dipeptide–, and amino acid–PQ derivatives. They were needed as references in the HPLC systems for the identification of the PQ-containing cleavage products, obtained during the lysosomal incubations of the tetrapeptide–PQ derivatives. Some of them were also used in separate incubation studies to confirm the degradation pattern of the different tetrapeptide–PQ derivatives.

Experimental Section

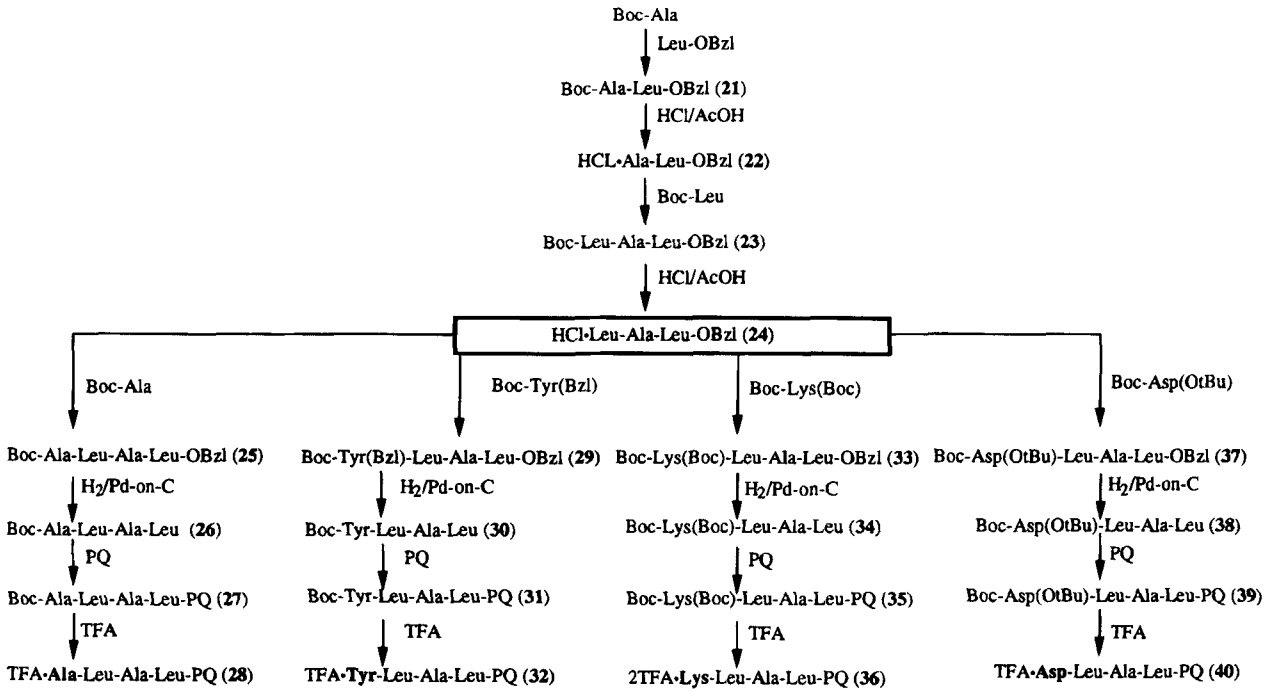
Materials—1-Hydroxybenzotriazole (HOBt) was from Fluka Chemie AG (Buchs, Switzerland); *N*,*N*′-dicyclohexylcarbodiimide (DCC), *N*-methylmorpholine, triethylamine (Et_3N), and diethylamine (Et_2NH) were from Merck (Darmstadt, Germany); 1,1′-carbonyldiimidazole (CDI) was from Merck and Sigma (St. Louis, MO); and *N*-(*tert*-butyloxycarbonyl)-ϵ-aminocaproic acid (Boc-EACA) was from Serva Feinbiochemica (Heidelberg, Germany). Primaquine [8-[(4-amino-1-methylbutyl)amino]-6-methoxyquinoline; PQ], as the diphosphate salt, was from Janssen Chimica (Beerse, Belgium). The following compounds were from Bachem Feinchemikalien AG (Bubendorf, Switzerland): *N*-(*tert*-butyloxycarbonyl)-L-leucine (Boc-Leu), 4-(tolylsulfonyl)-L-leucine benzyl ester (Tos-Leu-OBzl), 4-(tolylsulfonyl)-L-alanine benzyl ester (Tos-Ala-OBzl), *N*-(*tert*-butyloxycarbonyl)-L-alanine (Boc-Ala), 4-(tolylsulfonyl)-L-tyrosine benzyl ether benzyl ester [Tos-Tyr(Bzl)-OBzl], *N*-(*tert*-butyloxycarbonyl)-L-tyrosine benzyl ether [Boc-Tyr(Bzl)], α-*N*-(*tert*-butyloxycarbonyl-ϵ-*N*-(9-fluorenylmethyloxycarbonyl)-L-lysine [Boc-Lys(Fmoc)], *N*-(*tert*-butyloxycarbonyl)-L-aspartic acid β-benzyl ester [Boc-Asp(OBzl)], *N*-(*tert*-butyloxycarbonyl)-L-aspartic acid β-*tert*-butyl ester [Boc-Asp(OtBu)], *N*-(*tert*-butyloxycarbonyl)-ϵ-*N*-(*tert*-butyloxycarbonyl)-L-lysine [Boc-Lys(Boc)]. The

⊗ Abstract published in *Advance ACS Abstracts*, October 1, 1994.

0022-3549/95/3184-0249$09.00/0



Scheme 1—Summary of the syntheses of compounds **1–20**. The numbers given in parentheses are used in the Experimental Section, where further details about methods, reaction conditions, and analytical procedures can be found.

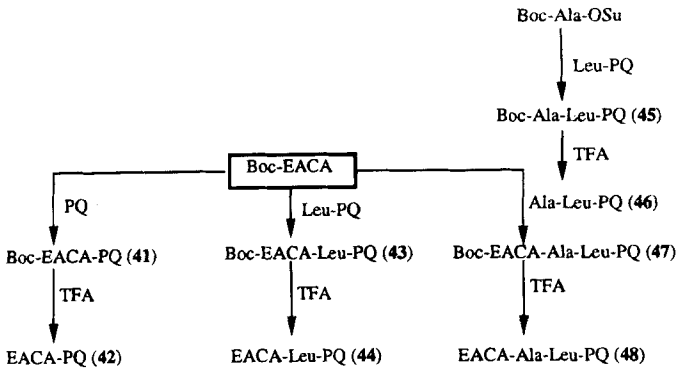


Scheme 2—Summary of the syntheses of compounds **21–40**. The numbers given in parentheses are used in the Experimental Section, where further details about methods, reaction conditions, and analytical procedures can be found.

reagents for the Edman degradation reaction were of sequential grade and bought from Sigma: Phenyl isothiocyanate (PITC), pyridine, trifluoroacetic acid (TFA), *n*-butyl acetate, ethyl acetate, and heptane. Dicarboxidine [γ,γ'-[4,4'-diamino-3,3'-(biphenylylenedioxy)]dibutyric acid dichloride] was from Sigma. Ninhydrin (1,2,3-indantrione monohydrate) was from Fluka. Leu-PQ, for the synthesis of **43** and **45**, was prepared in our laboratory from Boc-Leu-PQ as described for **42**. All the other reagents (solvents and salts) were of analytical grade. Acetonitrile of HPLC grade was bought from Merck and Fisons Scientific Equipment (Loughborough, England). *N,N*-Dimethyloctylamine (DMOA)-pro analysi was from Janssen.

General Synthetic Methods—When not stated otherwise, all reactions were performed at room temperature. PQ and PQ-containing derivatives were protected from light during all the syntheses. The syntheses were performed with no intention to optimize the reaction conditions.

Activation and Coupling Reactions—*Method I (DCC/HOBt)*[11]—A solution of the carboxyl component (1 equiv) and HOBt (2 equiv) in dry dimethylformamide (DMF) was cooled in an ice bath for 15 min and then treated with a solution of DCC (1.2 equiv) in dry DMF. The mixture was stirred for 5 min at 0 °C and then for 20–60 min at room temperature. The precipitated dicyclohexylurea (DCU) was



Scheme 3—Summary of the syntheses of compounds **41–48**. The numbers given in parentheses are used in the Experimental Section, where further details about methods, reaction conditions, and analytical procedures can be found.

filtered off and washed with DMF. The combined filtrates were added to the solution of the amino component (1.2 equiv) in DMF. In some cases, during the first hour of the synthesis, Et_3N was gradually added to give pH 7.5–9 to moist pH paper. The reaction mixture was stirred for 24–96 h. The progress of the reaction was monitored by TLC. After completion, the reaction mixture was filtered and purified as described in the text for each compound.

Method II (DCC)[12]—A solution of the carboxyl component (1–1.2 equiv) in CH_2Cl_2 was cooled in an ice bath and treated with a solution of DCC (1–1.2 equiv) in CH_2Cl_2. The mixture was stirred for 30 min and the precipitated DCU was filtered off. The filtrate was added to a solution of the amino component (1 equiv) in CH_2Cl_2. Et_3N (1–1.1 equiv) or *N*-methylmorpholine (1–1.1 equiv) was gradually added, dropwise, to give pH 7.5–9 to moist pH paper. The reaction mixture was stirred for ca. 20 h, filtered, and evaporated to dryness. The final product was isolated and purified as described in the text for each compound.

Method III (CDI)[13]—The carboxyl component (1 equiv) and CDI (1 equiv) were dissolved in dry DMF and the mixture was stirred for 30 min. The amino component (1 equiv), dissolved in dry DMF, was added and the reaction mixture was stirred overnight in the dark. The mixture was then diluted with an equal volume of 0.2 M Na_2CO_3, pH 11, and extracted with ether. The combined organic extracts were washed with 0.2 M citric acid, pH 2, dried over Na_2SO_4 and evaporated to dryness under reduced pressure.

Removal of Protecting Groups—*Method IV (Boc Removal by HCl/Acetic Acid)*[14]—The Boc-peptide as benzyl ester was dissolved in 1 M HCl/acetic acid (AcOH) and left for 40 min in the dark. The solvent was evaporated and the product (as hydrochloride) dried in vacuo over NaOH pellets. The deblocking reaction was checked by TLC.

Method V (Boc and OtBu Removal by TFA)[15,16]—Boc-tetrapeptide–PQ was dissolved in 100% TFA and left for ca. 1 h in the dark. Then TFA was evaporated in vacuo. The deblocking reaction was followed by TLC. In some cases the TFA salt was converted to the free base.

Method VI (OBzl and Bzl Removal by H_2/Pd-on-C)[17]—A solution of the OBzl- or Bzl-protected compound in MeOH was prepared in a closed flask provided with magnetic stirrer and gas inlet/outlet tubes. The air was removed by a slow stream of nitrogen and 10% palladium-on-charcoal was added. Nitrogen was introduced again for a few minutes and then a slow stream of hydrogen was supplied over the system for 3–10 h. Periodically, MeOH was added because of its evaporation. The progress of the reaction was followed by TLC. After completion of the reaction, the catalyst was removed by filtration and the filtrate evaporated in vacuo.

Reaction for Removal of the N-Terminal Amino Acid—*Edman Degradation*[18]—Tetrapeptide–PQ was dissolved in 0.1 mL of 1% PITC in 50% aqueous pyridine. The reaction mixture was flushed with N_2 for 20 s, and the tube was sealed and incubated for 2 h in 50 °C water bath, with occasional shakings. The reaction mixture, containing the phenylthiocarbamyl-tripeptide–PQ, was washed six times with 0.4 mL of heptane/ethyl acetate (10:1 and/or 1:2) to extract the excess PITC. The water phase was further dried under vacuo at room temperature for 20 min.

100% TFA was added and the sealed tube kept for 15 min in a 50 °C water bath. TFA was evaporated in vacuo and the residue dissolved in 0.1 N HCl. The tube was placed in an ice bath and the anilinothiazolinone was extracted five times with 0.4 mL of ice-cold *n*-butyl acetate saturated with water.

The water phase was made basic (pH 14) by dropwise addition of 1 M NaOH and extracted five times with 0.6 mL of ethyl acetate. The final product, the tripeptide–PQ, was either extracted to the ethyl acetate phase or stayed in the basic water phase, which was further evaporated to dryness in vacuo to give a thin film. At each step of the reaction, samples from both water and the organic phases were checked by TLC.

The tripeptide–PQ derivatives formed were used as reference samples in the HPLC. Part of the products was further subjected to a second Edman degradation to get the respective dipeptide–PQ derivatives. They were also used as references in the HPLC.

General Analytical Methods. Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC)—The HPLC system consisted of a HP 1090 chromatograph (Hewlett-Packard) with a Spherisorb 25 cm × 4.6 mm i.d., S5 ODS_2 column (Phase Separations Inc.) and a Lichrosorb RP 18 precolumn (Merck) and a 3392A integrator (Hewlett-Packard). The flow rate was 1 mL/min and the injection volume 20 μL. The absorbance was followed at 254 nm. The lowest detectable concentration of PQ was 0.8 μg/mL.

The mobile phases used for all derivatives (except EACA-peptides) were I, 0.025 M $NaH_2PO_4 \cdot H_2O$, pH 3 (600 mL), and acetonitrile (400 mL); II, 0.025 M $NaH_2PO_4 \cdot H_2O$, pH 3 (700 mL), and acetonitrile (300 mL); III, 0.025 M $NaH_2PO_4 \cdot H_2O$, pH 4 (600 mL), and acetonitrile (400 mL); IV, 0.025 M $NaH_2PO_4 \cdot H_2O$, pH 3 (750 mL), and acetonitrile (250 mL); and V, 0.025 M $NaH_2PO_4 \cdot H_2O$, pH 3.6 (700 mL), and acetonitrile (300 mL). All phases contained 0.6 mM DMOA.

The mobile phases used for the EACA-peptides were VI, 0.025 M $NaH_2PO_4 \cdot H_2O$, pH 3 (400 mL), and acetonitrile (600 mL); VII, 0.025 M $NaH_2PO_4 \cdot H_2O$, pH 3 (500 mL), acetonitrile (500 mL), and 0.75 mM DMOA; and VIII, 0.025 M $NaH_2PO_4 \cdot H_2O$, pH 3 (500 mL), acetonitrile (500 mL), and 0.25 mM DMOA.

Thin-Layer Chromatography (TLC)—TLC plates silica gel 60 F_{254} with layer thickness 0.25 mm from Merck were used. The mobile phases were A, chloroform:methanol:acetic acid (45:5:2); B, chloroform:methanol (10:1); C, chloroform:methanol (10:3); D, methylene chloride:acetone:acetic acid (40:10:1); E, methylene chloride:methanol (9:1); F, toluene:acetonitrile (2:1); G, methylene chloride:acetone (4:1); H, *n*-butanol:acetic acid:water (4:1:1); I, methylene chloride:acetone (3:1); and J, ethyl acetate:acetone:acetic acid:water (5:3:1:1).

The spots were detected under UV light and by either ninhydrin or Cl_2/dicarboxidine development.

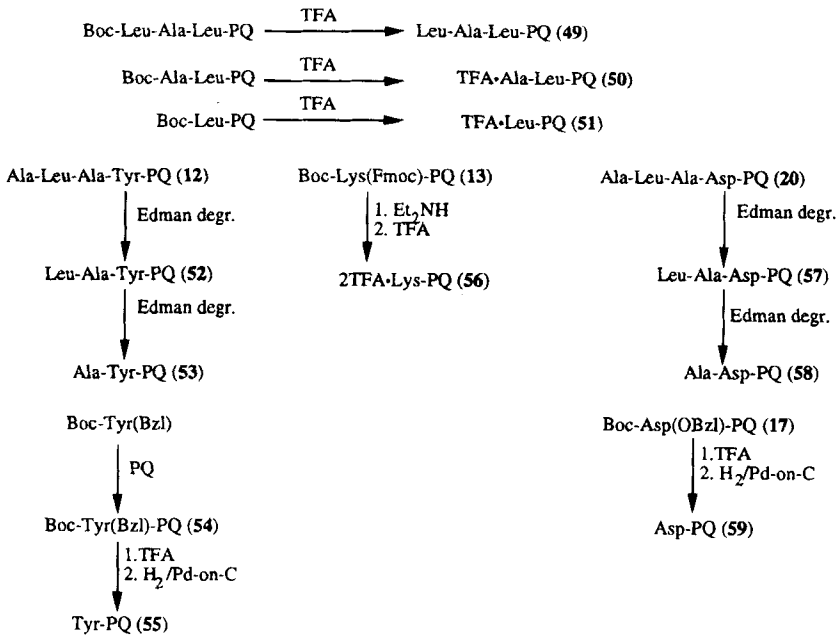
Physicochemical determinations—The melting point values are uncorrected. The elemental analyses were done on a Carlo Erba Model 1106 analyzer. The optical rotations were measured with a Perkin-Elmer polarimeter. ^{1}H-NMR analyses were performed on Varian Unity 300 MHz instrument. Amino acid analyses were made using a LKB-4151 Alpha Plus amino acid analyzer (Pharmacia) at the Amino Acid Analyses Laboratory at the Biomedical Center, Uppsala, Sweden.

Boc-Leu-Ala-OBzl (1) was prepared from Boc-Leu (2.5 g, 10 mmol) in 10 mL of CH_2Cl_2, DCC in 2 mL of CH_2Cl_2, a suspension of Tos-Ala-OBzl in 6 mL of CH_2Cl_2, and *N*-methylmorpholine for 20 h according to method II. The oily residue was dissolved in 100 mL of ethyl acetate, filtered, and washed with 1 M $KHSO_4$, brine, 1 M $NaHCO_3$, and brine. The organic layer after being dried over anhydrous Na_2SO_4, filtered, and evaporated, gave an oily residue. Yield: 97%. R_f (B) = 0.89.

HCl·Leu-Ala-OBzl (2) was prepared from Boc-Leu-Ala-OBzl (3.8 g, 9.7 mmol) dissolved in 50 mL of 1 M HCl/AcOH according to method IV. Yield: 98% of an oil. R_f (B) = 0.25.

Boc-Ala-Leu-Ala-OBzl (3) was prepared from Boc-Ala (2.3 g, 12 mmol), DCC, HCl·Leu-Ala-OBzl, and Et_3N according to method II. The crude product was crystallized from ethyl ether:petroleum ether (1:3) to give a white powder. Yield: 52%. R_f (B) = 0.92. Mp 143–147 °C. $[\alpha]^{23}_D$ = −63.6° (*c* = 1, MeOH). Anal. Calcd for $C_{24}H_{57}N_3O_6$: 62.18% C, 12.4% H, 9.06% N. Found: 62.3% C, 12.2% H, 8.89% N.

Boc-Ala-Leu-Ala (4) was prepared from Boc-Ala-Leu-Ala-OBzl (2.3 g, 5 mmol) dissolved in 35 mL of MeOH and hydrogenated over 0.23 g of Pd-on-C according to method VI. Evaporation of the solvent gave an oily residue which solidified after drying in vacuo. Yield:



Scheme 4—Summary of the syntheses of compounds **49–59**. The numbers given in parentheses are used in the Experimental Section, where further details about methods, reaction conditions, and analytical procedures can be found.

94% of white crystals. R_f (B) = 0.06. Anal. Calcd for $C_{17}H_{31}N_3O_6$: 54.67% C, 8.37% H, 11.25% N. Found: 54.24% C, 8.58% H, 11.01% N.

Boc-Ala-Leu-Ala-Leu-OBzl (5) was prepared from Boc-Ala-Leu-Ala (1.9 g, 5 mmol) and HOBt in 8 mL of DMF, DCC in 2 mL of DMF and a suspension of Tos-Leu-OBzl in 4 mL of DMF for 24 h according to method I. The reaction mixture was filtered and after the addition of 100 mL of ethyl acetate washed three times with 1 M $KHSO_4$, 1 M $NaHCO_3$, and brine. After evaporation of solvent, the residue was dissolved in 15 mL of EtOH and precipitated by adding 100 mL of 1 M $KHSO_4$. The resulting oil was extracted into ethyl acetate. The organic layer was washed with brine, dried over $MgSO_4$, filtered, and evaporated. Yield: 61% of white crystals. R_f (B) = 0.74. Mp 184–187 °C $[\alpha]^{23}_D = -80.7°$ (c = 1, MeOH). Anal. Calcd for $C_{30}H_{48}N_4O_7$: 62.48% C, 8.38% H, 9.71% N. Found: 62.1% C, 8.04% H, 9.4% N.

Boc-Ala-Leu-Ala-Leu (6) was prepared from Boc-Ala-Leu-Ala-Leu-OBzl according to method VI. Yield: 98% of a white powder. R_f (C) = 0.20. $[\alpha]^{23}_D = -67.6°$ (c = 1, MeOH).

Boc-Ala-Leu-Ala-Leu-PQ (7) was prepared from Boc-Ala-Leu-Ala-Leu (0.4 g, 0.8 mmol) and HOBt in 9 mL of DMF, DCC in 2 mL of DMF, and PQ in 3 mL of DMF for 72 h according to method I. Ethyl acetate (150 mL) was added to the reaction mixture, which was then washed with 0.2 M citric acid, pH 2, 1 M $NaHCO_3$, and brine. The organic layer was dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness. Yield: 18% of light yellow powder. R_f (A) = 0.66.

TFA·Ala-Leu-Ala-Leu-PQ (8) was prepared from Boc-Ala-Leu-Ala-Leu-PQ (7.6 mg, 0.1 mmol) according to method V. Yield: 98% of a yellow film. R_f (A) = 0.05, R_f (H) = 0.35, R_f (C) = 0.10. t_R (I) = 17.62 min.

Boc-Ala-Leu-Ala-Tyr(Bzl)-OBzl (9) was prepared from Boc-Ala-Leu-Ala (3.7 g, 10 mmol), Tos-Tyr(Bzl)-OBzl, DCC, HOBt, and Et_3N according to method I. Yield: 34% of white crystals. R_f (B) = 0.74. Mp 193–196 °C. $[\alpha]^{23}_D = -47.3°$ (c = 1, MeOH). Anal. Calcd for $C_{40}H_{52}N_4O_8$: 67.02% C, 7.31% H, 7.82% N. Found: 66.68% C, 7.51% H, 7.72% N.

Boc-Ala-Leu-Ala-Tyr (10) was prepared from Boc-Ala-Leu-Ala-Tyr(Bzl)-OBzl according to method VI. Yield: 98% of a white powder. R_f (C) = 0.19. $[\alpha]^{23}_D = -51.3°$ (c = 1, MeOH).

Boc-Ala-Leu-Ala-Tyr-PQ (11) was prepared from Boc-Ala-Leu-Ala-Tyr (0.4 g, 0.7 mmol) and HOBt in 25 mL of DMF, DCC in 3 mL of DMF, and PQ in 3 mL of DMF for 96 h according to method I. Ethyl acetate (250 mL) was added to the reaction mixture, which was then washed with 0.2 M citric acid, pH 2, 1 M $NaHCO_3$, and brine. The organic layer was dried over K_2CO_3, filtered, and evaporated to dryness. Yield: 72% of a light yellow powder. R_f (A) = 0.55.

TFA·Ala-Leu-Ala-Tyr-PQ (12) was prepared from Boc-Ala-Leu-Ala-Tyr-PQ (1.5 mg, 1.93×10^{-3} mmol) according to method V. Yield: 98% of a yellow film. R_f (A) = 0.06, R_f (C) = 0.11. t_R (I) = 9.54 min, main peak.

Boc-Lys(Fmoc)-PQ (13) was prepared from Boc-Lys(Fmoc) (185 mg, 0.4 mmol) in 1 mL of DMF, HOBt in 0.7 mL of DMF, DCC in 0.6 mL of DMF, and PQ in 0.7 mL of DMF for 29 h according to method I. The reaction mixture was evaporated to dryness, redissolved in 200 mL of ethyl ether, and washed three times with 1 M $NaHCO_3$ and brine. The organic layer was dried over anhydrous $MgSO_4$, filtered, and evaporated to dryness. Yield: 76% of a light yellow powder. R_f (D) = 0.47.

Lys(Fmoc)-PQ (14)—Boc-Lys(Fmoc)-PQ (0.174 g, 0.25 mmol) was dissolved in 2.5 mL of CH_2Cl_2, and TFA (1.25 mL) was added dropwise at 0 °C under nitrogen. After stirring for 3 h in the dark, the reaction mixture was quenched in an ice-cold mixture of 30% aqueous K_2CO_3 (20 mL) and CH_2Cl_2 (10 mL) with rapid stirring. The yellow organic layer was separated and saved. The aqueous layer was extracted twice with CH_2Cl_2. The combined organic phases were then washed with brine, dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness. Yield: 96% of a light yellow powder. R_f (E) = 0.18.

Boc-Ala-Leu-Ala-Lys(Fmoc)-PQ (15) was prepared from Boc-Ala-Leu-Ala (88 mg, 0.23 mmol) in 0.4 mL of DMF, HOBt in 0.4 mL of DMF, DCC in 0.4 mL of DMF, and Lys-PQ in 0.5 mL of DMF for 21 h according to method I. The reaction mixture was evaporated to dryness, redissolved in 200 mL of ethyl acetate, and washed three times with 1 M $NaHCO_3$ and brine. The organic layer was dried over anhydrous $MgSO_4$, filtered, and evaporated to dryness. Yield: 53% of a light yellow powder. R_f (D) = 0.27.

Ala-Leu-Ala-Lys-PQ (16) was prepared from Boc-Ala-Leu-Ala-Lys(Fmoc)-PQ (9.0 mg, 9.32×10^{-3} mmol) according to method V. The intermediate product TFA·Ala-Leu-Ala-Lys(Fmoc)-PQ (15 mg), having R_f (E) = 0.12 and R_f (H) = 0.59, was dissolved in 1 mL of DMF and treated with 0.1 mL of Et_2NH for 4 h to remove the Fmoc protecting group.[19] The Et_2NH and DMF were evaporated in vacuo. A brown oil was obtained, consisting of the final product, the byproduct dibenzofulvene, and traces of still-blocked TFA·Ala-Leu-Ala-Lys(Fmoc)-PQ, as proved by TLC (systems E and H). To complete the deblocking, an additional 8 h treatment with 0.25 mL of Et_2NH in DMF was done. The solvents were evaporated in vacuo, and the residue was triturated seven times with 1.5 mL of diethyl ether to remove the dibenzofulvene. Yield: 60% of a yellow film. R_f (H) = 0.23. t_R (I) = 4.26 min, t_R (IV) = 14.86 min.

Boc-Asp(OBzl)-PQ (17) was prepared from Boc-Asp(OBzl) (1.3 g, 4 mmol), HOBt, DCC, and PQ according to method I. Yield: 52% of a yellow sticky foam. R_f (D) = 0.67.

Asp(OBzl)-PQ (18) was prepared from Boc-Asp(OBzl)-PQ according to method V. Yield: 89% of a light yellow powder. R_f (D) = 0.02.

Boc-Ala-Leu-Ala-Asp(OBzl)-PQ (19) was prepared from Boc-Ala-Leu-Ala (53 mg, 0.14 mmol), HOBt, DCC, and Asp(OBzl)-PQ according to method I. The reaction mixture was evaporated to dryness, redissolved in 200 mL of ethyl acetate, and washed three times with 1 M $NaHCO_3$ and brine. The organic layer was dried over anhydrous $MgSO_4$, filtered, and evaporated to dryness. Yield: 78% of a yellowish, sticky solid. R_f (D) = 0.18.

TFA·Ala-Leu-Ala-Asp-PQ (20) was prepared from Boc-Ala-Leu-Ala-Asp(OBzl)-PQ (19 mg, 0.023 mmol) in 10 mL of MeOH and hydrogenated over 0.1 g of 10% Pd-on-C for 3 h according to method VI. Yield: 87% of pure intermediate product Boc-Ala-Leu-Ala-Asp-PQ. R_f (D) = 0.02. 100% TFA (0.25 mL) was added to Boc-Ala-Leu-Ala-Asp-PQ (1.5 mg, 2.05 × 10^{-3} mmol) for 30 min according to Method V. TFA was evaporated in vacuo and traces removed by freeze drying for 30 min. Yield: 90% of a yellow film. R_f (D) = 0. t_R (I) = 5.82 min, t_R (II) = 15.18 min, t_R (IV) = 34.35 min, t_R (V) = 8.0 min.

Boc-Ala-Leu-OBzl (21) was prepared from Boc-Ala (1.9 g, 10 mmol), DCC, Tos-Leu-OBzl, and *N*-methylmorpholine according to method II. The crude product contained a substantial amount of Leu-OBzl. It was dissolved in 20 mL of EtOH and precipitated by 100 mL of 1 M $KHSO_4$. The resulting oil was extracted with ethyl ether and the organic layer was washed with 1 M $KHSO_4$ and brine. Evaporation of the solvent gave an oil, which failed to crystallize. Yield: 92%. R_f (B) = 0.93. Anal. Calcd for $C_{21}H_{32}N_2O_5$: 64.26% C, 8.22% H, 7.14% N. Found: 64.02% C, 8.43% H, 7.40% N.

HCl·Ala-Leu-OBzl (22) was prepared from Boc-Ala-Leu-OBzl (3.6 g, 9.2 mmol) according to method IV. Yield: 98% of a yellow oil. R_f (B) = 0.30.

Boc-Leu-Ala-Leu-OBzl (23) was prepared from Boc-Leu (2.74 g, 11 mmol), DCC, HCl·Ala-Leu-OBzl, and Et_3N according to method II. Yield: 87% of an oil. R_f (B) = 0.82.

HCl·Leu-Ala-Leu-OBzl (24) was prepared from Boc-Leu-Ala-Leu-OBzl (5.06 g, 10 mmol) dissolved in 60 mL of 1 M HCl/AcOH according to method IV. Yield: 95%.

Boc-Ala-Leu-Ala-Leu-OBzl (25) was prepared from Boc-Ala (1.7 g, 9 mmol), DCC, HCl·Leu-Ala-Leu-OBzl, and Et_3N according to method II. Yield: 52% of white crystals. R_f (B) = 0.74. Mp 184–187 °C. $[\alpha]^{23}_D$ = −80.3° (*c* = 1, MeOH). Anal. Calcd for $C_{30}H_{48}N_4O_7$: 62.48% C, 8.38% H, 9.71% N. Found: 62.1% C, 8.04% H, 9.4% N.

Boc-Ala-Leu-Ala-Leu (26) is identical with compound **6**.

Boc-Ala-Leu-Ala-Leu-PQ (27)—The compound was identical with compound **7**.

TFA·Ala-Leu-Ala-Leu-PQ (28)—The compound was identical with compound **8**.

Boc-Tyr(Bzl)-Leu-Ala-Leu-OBzl (29) was prepared from Boc-Tyr(Bzl) (4.83 g, 13 mmol), DCC, HCl·Leu-Ala-Leu-OBzl, and Et_3N according to method II. The crude product after crystallization from ethyl acetate:ethyl ether:petroleum ether (1:1:5) gave white crystals. Yield: 52%. R_f (B) = 0.79. Mp 137–148 °C. $[\alpha]^{23}_D$ = −42.9° (*c* = 1, MeOH). Anal. Calcd for $C_{43}H_{58}N_4O_8$: 68.05% C, 7.70% H, 7.38% N. Found: 67.81% C, 7.92% H, 7.13% N.

Boc-Tyr-Leu-Ala-Leu (30) was prepared from Boc-Tyr(Bzl)-Leu-Ala-Leu-OBzl according to method VI. Yield: 97% of a white powder. R_f (C) = 0.23. $[\alpha]^{23}_D$ = −49.2° (*c* = 1, MeOH).

Boc-Tyr-Leu-Ala-Leu-PQ (31) was prepared from Boc-Tyr-Leu-Ala-Leu (0.6 g, 1 mmol) and HOBt in 6.5 mL of DMF, DCC in 2 mL of DMF, and Et_3N and PQ in 6 mL of DMF for 96 h according to method I. Ethyl acetate (16 mL) was added to the reaction mixture, which was then washed with 0.2 M citric acid, pH 2, 1 M $NaHCO_3$, and brine. The organic layer was dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness. Yield: 31% of a yellow powder.

TFA·Tyr-Leu-Ala-Leu-PQ (32) was prepared from Boc-Tyr-Leu-Ala-Leu-PQ (0.93 mg, 1.1 × 10^{-3} mmol) according to method V. Yield: 96% of a yellow film. R_f (E) = 0.06. t_R (I) = 7.42 min, main peak.

Boc-Lys(Boc)-Leu-Ala-Leu-OBzl (33) was prepared from Boc-Lys(Boc) (6.32 g, 12 mmol), DCC, Et_3N, and HCl·Leu-Ala-Leu-OBzl according to method II. The crude product, after crystallizing from ethyl acetate:ethyl ether:petroleum ether (1:1:5) twice, gave a white powder. Yield: 42%. R_f (B) = 0.86. Mp 199–202 °C. $[\alpha]^{23}_D$ = −60.1° (*c* = 1, MeOH). Anal. Calcd for $C_{38}H_{63}N_5O_9$: 62.19% C, 8.65% H, 9.54% N. Found: 61.87% C, 8.91% H, 9.18% N.

Boc-Lys(Boc)-Leu-Ala-Leu (34) was prepared from Boc-Lys(Boc)-Leu-Ala-Leu-OBzl according to method VI. Yield: 96% of a white powder. R_f (C) = 0.22. $[\alpha]^{23}_D$ = −53.9° (*c* = 1, MeOH).

Boc-Lys(Boc)-Leu-Ala-Leu-PQ (35) was prepared from Boc-Lys-(Boc)-Leu-Ala-Leu (0.7 g, 1.0 mmol) and HOBt in 5.5 mL of DMF, DCC in 2 mL of DMF, and Et_3N and PQ in 2 mL of DMF for 96 h according to method I. Ethyl acetate (100 mL) was added to the reaction mixture, which was then washed with 1 M $KHSO_4$, 1 M $NaHCO_3$, and brine. The organic layer was dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness. The residue was further purified by adsorption chromatography. Yield: 68% of a yellow powder. R_f (A) = 0.64.

2TFA·Lys-Leu-Ala-Leu-PQ (36) was prepared from Boc-Lys(Boc)-Leu-Ala-Leu-PQ (0.05 g, 0.06 mmol) according to method V. Yield: 97% of a yellow film. R_f (B) = 0.36. t_R (I) = 12.48 min.

Boc-Asp(OtBu)-Leu-Ala-Leu-OBzl (37) was prepared from Boc-Asp(OtBu) (6 g, 13 mmol), DCC, HCl·Leu-Ala-Leu-OBzl, and Et_3N according to method II. The crude product after crystallization from ethyl acetate:ethyl ether:petroleum ether (1:1:5) gave white powder. Yield: 34.1%. R_f (B) = 0.82. Mp 167–169 °C. $[\alpha]^{23}_D$ = −64.3° (*c* = 1, MeOH). Anal. Calcd for $C_{35}H_{56}N_4O_9$: 62.11% C, 8.34% H, 8.28% N. Found: 62.02% C, 8.13% H, 8.34% N.

Boc-Asp(OtBu)-Leu-Ala-Leu (38) was prepared from Boc-Asp-(OtBu)-Leu-Ala-Leu-OBzl according to method VI. Yield: 98% of a white powder. R_f (C) = 0.25. $[\alpha]^{23}_D$ = −60.8° (*c* = 1, MeOH).

Boc-Asp(OtBu)-Leu-Ala-Leu-PQ (39) was prepared from Boc-Asp(OtBu)-Leu-Ala-Leu (0.2 g, 0.34 mmol) and HOBt in 2 mL of DMF, DCC in 0.6 mL of DMF, and Et_3N and PQ in 2 mL of DMF for 26 h according to method I. Ethyl acetate (40 mL) was added to the reaction mixture, which was then washed with 1 M $KHSO_4$, 1 M $NaHCO_3$, and brine. The organic layer was dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness. Yield: 44% of a yellow powder. R_f (B) = 0.64.

TFA·Asp-Leu-Ala-Leu-PQ (40) was prepared from Boc-Asp-(OtBu)-Leu-Ala-Leu-PQ (20 mg, 0.02 mmol) according to method V. Yield: 96% of a yellow film. R_f (A) = 0.06, R_f (B) = 0.11. t_R (I) = 12.56 min, t_R (II) = 50 min, t_R (III) = 10.50 min.

Boc-EACA-PQ (41) was prepared from Boc-EACA (2.45 g, 10.6 mmol), CDI, and PQ dissolved in DMF according to method III. The mixture was then diluted with an equal volume of 0.2 M Na_2CO_3, pH 11, and extracted with ether. The combined organic extracts were washed with 0.2 M citric acid, pH 2, dried over Na_2SO_4, and evaporated to dryness. Yield: 50%. R_f (A) = 0.62.

EACA-PQ (42) was prepared from Boc-EACA-PQ (30 mg, 0.06 mmol) dissolved in 1 mL of 100% TFA and left for 30 min in the dark. TFA was then evaporated, and the resulting oil was dissolved in 0.2 M $NaHCO_3$, pH 11, and extracted into ethyl acetate as a free base. The combined organic extracts were dried over Na_2SO_4, filtered, and evaporated to dryness. Yield: 63%. R_f (A) = 0.19. t_R (VI) = 5.22 min.

Boc-EACA-Leu-PQ (43) was prepared from Boc-EACA (0.86 g, 3.7 mmol), CDI, and Leu-PQ dissolved in DMF according to method III. The mixture was then diluted with an equal volume of 0.2 M $NaCO_3$, pH 11, and extracted with ether. The combined organic extracts were washed with 0.2 M citric acid, pH 2, dried over Na_2SO_4, filtered, and evaporated to dryness. Yield: 25%. R_f (A) = 0.66.

EACA-Leu-PQ (44) was prepared from Boc-EACA-Leu-PQ (45 mg, 0.08 mmol) as described for compound **42**. Yield: 64%. R_f (A) = 0.23. t_R (VIII) = 7.98 min.

Boc-Ala-Leu-PQ (45)—Boc-Ala-OSu (0.31 g, 1.08 mmol), Leu-PQ (0.45 g, 1.20 mmol), and $KHCO_3$ (0.24 g, 2.40 mmol) were dissolved in a mixture of 5 mL of water and 8 mL of dioxane and was stirred for 48 h in the dark. Citric acid (0.2 M, pH 2) was added and the product was extracted into ethyl acetate. The combined organic phases were dried over Na_2SO_4, filtered, and evaporated to dryness. Yield: 52%. R_f (A) = 0.72.

Ala-Leu-PQ (46) was prepared from Boc-Ala-Leu-PQ (0.31 g, 0.57 mmol) as described for compound **42**. Yield: 92%. R_f (A) = 0.21.

Boc-EACA-Ala-Leu-PQ (47) was prepared from Boc-EACA (0.11 g, 0.45 mmol), HOBt, DCC, and Ala-Leu-PQ dissolved in DMF overnight according to method I. The DCU formed was removed by filtration and the filtrate was evaporated under reduced pressure. The residue was dissolved in ethyl acetate and washed with 0.2 M

Na_2CO_3, pH 11, and 0.2 M sodium citrate, pH 2. The organic layer was dried over Na_2SO_4, filtered, and evaporated to dryness. R_f (A) = 0.62.

EACA-Ala-Leu-PQ (48) was prepared from Boc-EACA-Ala-Leu-PQ (0.12 g, 0.18 mmol) as described for compound **42**. Yield: 14%. R_f (A) = 0.19. t_R (VII) = 4.82 min.

Leu-Ala-Leu-PQ (49) was prepared from Boc-Leu-Ala-Leu-PQ (0.05 g, 0.08 mmol) according to method V. Yield: 96% of a yellow film. t_R (I) = 19.34 min, t_R (III) = 27.30 min.

TFA·Ala-Leu-PQ (50) was prepared from Boc-Ala-Leu-PQ (0.40 g, 0.74 mmol) according to method V. Yield: 98% of a yellow film. t_R (I) = 11.08 min, t_R (III) = 15.58 min.

TFA·Leu-PQ (51) was prepared from Boc-Leu-PQ (0.2 g, 0.46 mmol) according to method V. Yield: 99% of a yellow film. t_R (I) = 12.19 min, t_R (III) = 19.36 min.

Leu-Ala-Tyr-PQ (52) was prepared from TFA·Ala-Leu-Ala-Tyr-PQ (7 mg, 8.77×10^{-3} mmol) according to the Edman degradation. The phenylthiocarbamyl(PTC)-Ala-Leu-Ala-Tyr-PQ was washed once with heptane:ethyl acetate (10:1) and once with heptane:ethyl acetate (1:2). The anilinothiazolinone(ATZ)-Ala formed was washed out three times with *n*-butyl acetate and the final produce Leu-Ala-Tyr-PQ was extracted into the ethyl acetate phase. All TLC/s were run in system A. Yield: 23% of a yellow film. R_f (A) = 0.04. t_R (I) = 10.18 min, main peak.

Ala-Tyr-PQ (53) was prepared from Leu-Ala-Tyr-PQ (1.2 mg, 2.0×10^{-3} mmol) according to the Edman degradation. The PTC-Leu-Ala-Tyr-PQ was washed with heptane:ethyl acetate (10:1 and 1:2). The ATZ-Leu formed was washed out with *n*-butyl acetate and the final product, Ala-Tyr-PQ, was extracted into the ethyl acetate phase. Yield: 40% of a yellow film. R_f (A) = 0.05. t_R (I) = 6.88 min, main peak.

Boc-Tyr(Bzl)-PQ (54) was prepared from Boc-Tyr(Bzl) (15.7 mg, 0.04 mmol) and HOBt in 3 mL of DMF, DCC in 2 mL of DMF, and PQ in 2 mL of DMF for 40 h according to method I. Ethyl acetate (100 mL) was added to the reaction mixture, which was then washed with 1 M $KHSO_4$, 1 M $NaHCO_3$, and brine. The organic layer was dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness. Yield: 70% of a yellow powder. R_f (A) = 0.64.

Tyr-PQ (55)—Method V was applied to Boc-Tyr(Bzl)-PQ (20 mg, 0.03 mmol). The intermediate product, TFA·Tyr(OBzl)-PQ (Yield: 99% of a yellow film), had R_f (A) = 0.20, R_f (C) = 0.53, R_f (I) = 0.05, and method VI was further applied to it. The final product (Yield: 97% of a yellow film) had R_f (A) = 0.10, R_f (C) = 0.38, and t_R (I) = 7.49 min (main peak).

2TFA·Lys-PQ (56)—To remove the Fmoc protecting group, Boc-Lys(Fmoc)-PQ (11 mg, 0.015 mmol) was dissolved in 1 mL of dry DMF and treated with 0.1 mL of Et_2NH for 5.5 h. The Et_2NH and DMF were evaporated in vacuo. The residue was triturated 3 times with 1.5 mL of diethyl ether to remove the byproduct dibenzofulvene. The homogenicity of the intermediate product Boc-Lys-PQ (11.5 mg, 0.023 mmol) was checked in the TLC systems D, E, F, and H, and method V was applied to give the final product 2TFA·Lys-PQ. Yield: 92% of a yellow film. R_f (H) = 0.19. t_R (IV) = 6.20 min. The same results were obtained by reversing the order of the two steps.

Leu-Ala-Asp-PQ (57) was prepared from TFA·Ala-Leu-Ala-Asp-PQ (0.6 mg, 7.39×10^{-4} mmol) according to the Edman degradation. The PTC-Ala-Leu-Ala-Asp-PQ was washed with heptane:ethyl acetate (10:1). The ATZ-Ala formed was washed out with *n*-butyl acetate. The final product, Leu-Ala-Asp-PQ, stayed in the basic water phase after several extractions with ethyl acetate. Yield: 38% of a yellow film. R_f (J) = 0.38. t_R (II) = 12.96 min, t_R (V) = 12.77 min.

Ala-Asp-PQ (58) was prepared from Leu-Ala-Asp-PQ (2.8 mg, 5.03×10^{-3} mmol) according to the Edman degradation. The PTC-Leu-Ala-Asp-PQ was washed with heptane:ethyl acetate (10:1). The ATZ-Leu formed was washed out with *n*-butyl acetate. The final product, Ala-Asp-PQ, stayed in the basic water phase after several extractions with ethyl acetate. Yield: 45% of a yellow film. R_f (J) = 0.19. t_R (V) = 7.70 min.

Asp-PQ (59)—Method V was applied to Boc-Asp(OBzl)-PQ (13.4 mg, 0.024 mmol). The intermediate product, TFA·Asp(OBzl)-PQ (Yield: 97% of a yellow film), had R_f (D) = 0.02 and R_f (H) = 0.77, and method VI was further applied to it. The final product Asp-PQ (Yield: 96% of a yellow film) had R_f (H) = 0.30 and t_R (V) = 8.60 min.

Results

The tetrapeptide–PQ compounds were synthesized as shown and summarized in Schemes 1 and 2 and the EACA-PQ compounds as shown in Scheme 3. The reference substances, needed for identification purposes in the analytical chromatographic systems, were prepared as described in Scheme 4. Each separate intermediate and final product is identified by a number as specified in the schemes. The number is used in the detailed descriptions of the syntheses of the respective compounds.

The general methods, as described above, were used as far as possible. The reaction rate of each step, as well as the purification of each compound, was routinely followed by TLC and when appropriate by HPLC. Each product was characterized in different ways and relevant data are given in the text. Some key products were characterized by elemental and amino acid analyses.

Discussion

Targeting of drugs is today a widely accepted concept to experimentally improve the therapeutic efficacy of toxic substances or to circumvent physiological barriers. For instance, the entrapment of cytokines in liposomes or polyacryl starch microparticles[4] may be used to avoid systemic effects, if the carriers can be selectively directed to the wanted target. The covalent binding of antileishmanial drugs to polyacryl starch microparticles has been shown to significantly increase their uptake and therapeutic activity in the lysosomal vacuome of the Kupffer cells, where the *Leishmania* parasites proliferate in infected animals.[7,20] A crucial point for a successful exploitation of the concept is, however, that the drug can be released in an active form from the carrier, preferably in a controlled, predetermined way.

Drugs having a primary amino group, like primaquine (PQ), can be conveniently coupled to lysosomotropic carriers via polypeptide spacer arms. These are cleaved by the lysosomal enzymes so that the active drug can be subsequently released. In our earlier work,[5] we studied the release of PQ bound to starch microparticles via Ala-Leu peptides. The fastest release was obtained with the tetrapeptide Ala-Leu-Ala-Leu, and the microparticle conjugate was found to be active against *Leishmania* parasites both in vitro[6] and in vivo.[7] Different peptide spacer arms have been used to couple drugs also to serum albumin[21] and synthetic polymers.[22] The present work describes the synthesis of many PQ-derivatives like tetra-, tri- and dipeptide–PQ, amino acid–PQ, EACA–PQ, and several peptide intermediates. The synthesis of these compounds was performed in solution using the coupling reagents DCC and CDI. The bond between the drug PQ and the peptides was thus an amide bond. As a temporary protection we used in all cases Boc for the α-NH_2 groups and OBzl for the α-COOH groups. The semipermanent protection at the side-chain functions of Tyr, Lys, and Asp was necessary in order to avoid possible side reactions. We used Bzl for the phenolic group of the Tyr, Fmoc or Boc for the ϵ-NH_2 group of Lys, and OBzl or OtBu for the β-COOH group of Asp. The strategic plan for the synthesis was simplified by a core of a common tripeptide in each of the two series of tetrapeptides, but complicated by the sensitivity of PQ for light and high pH values. Thus, the synthetic schemes were designed to reduce the exposure of PQ to light. Moreover, PQ was normally condensed with the tetrapeptides by a DCC/HOBt reaction, but peptides **15** and **19** were prepared from the tripeptide with the appropriate Lys-PQ and Asp-PQ derivatives. This deviation from the normal strategy was found to give the best yield in the two cases.

Concerning the removal of protecting groups, we applied the most widely used procedures, namely, Boc removal by HCl/AcOH or TFA, OtBu removal by TFA, OBzl and Bzl removal by H_2/Pd-on-C, and Fmoc removal by Et_2NH. By stepwise removal of only the N-terminal residue from peptides (proteins), the Edman degradation can yield the entire amino acid sequence. However, we used it as a method to prepare the PQ–peptide with one amino acid less than the original.

All the reaction conditions were chosen to minimize racemization. Thus, in most of the syntheses, we added as an auxiliary nucleophile, HOBt, due to its superiority to HOSu.[23] The temperature was kept at 0 °C during the initial phases of the condensation steps, as chiral integrity is improved at lower temperatures.[24] However, DMF had to be used instead of nonpolar solvents in most of the reactions to improve the solubility of the peptide intermediates. Subsequent degradation with lysosomal enzymes indicated that no racemization had taken place as all the PQ–peptides obtained were totally degraded within experimental errors.[10] Only the Asp-containing peptides were cleaved too slowly to give useful information about the stereochemical purity, but this may be due to lack of such an enzyme specificity.

The reactions applied gave products which normally could be purified by washings with suitable solvent mixtures. However, derivative **35** was purified by adsorption chromatography due to its specific solubility profile. In all cases, derivatives were obtained which were shown to be homogeneous in analytical HPLC. In some cases, though, the derivatives may have been contaminated by traces of dicyclohexylurea, which however is of no significance in our studies.

In conclusion, the synthetic schemes developed in this paper are well-suited for the synthesis of all four groups of PQ derivatives.

References and Notes

1. Alving, C. R. *Parasitol. Today* **1986**, *2*, 101–107.
2. Edman, P.; Artursson, P.; Laakso, T.; Sjöholm, I. In *Polymers in Controlled Drug Delivery*; Illum, L.; Davis, S. S., Eds.; Wright: Bristol, 1987; pp 87–98.
3. Artursson, P.; Edman, P.; Sjöholm, I. *J. Pharmacol. Exp. Ther.* **1984**, *231*, 705–712.
4. Degling, L.; Stjärnkvist, P.; Sjöholm, I. *Pharm. Res.* **1993**, *10*, 783–790.
5. Laakso, T.; Stjärnkvist, P.; Sjöholm, I. *J. Pharm. Sci.* **1987**, *76*, 134–140.
6. Stjärnkvist, P.; Artursson, P.; Brunmark, A.; Laakso, T.; Sjöholm, I. *Int. J. Pharm.* **1987**, *40*, 215–222.
7. Stjärnkvist, P. *Int. J. Pharm.* **1993**, *96*, 23–32.
8. Novikoff, A. B.; Novikoff, P. M. In *The Liver–Biology and Pathobiology*; Arias, I., Popper, H., Jakoby, W., Schachter, D., Shafrits, D., Eds.; Raven Press: New York, 1988; p 227.
9. Kopeček, J. *Biomaterials* **1984**, *5*, 19–25.
10. Borissova, R.; Stjärnkvist, P.; Karlsson, M. O.; Sjöholm, I. *J. Pharm. Sci.*, following paper in this issue.
11. König, W.; Geiger, R. *Chem. Ber.* **1970**, *103*, 788–798.
12. Sheehan, J. C.; Hess, G. P. *J. Am. Chem. Soc.* **1955**, *77*, 1067–1068.
13. Staab, H. A. *Justus Liebigs Ann. Chem.* **1957**, *609*, 75–83.
14. Noda, K.; Terada, S.; Mitsuyasu, N.; Waki, M.; Kato, T.; Izumiya, N. *Naturwissenschaften* **1971**, *58*, 147–148.
15. Schnabel, E.; Klostermeyer, H.; Berndt, H. *Justus Liebigs Ann. Chem.* **1971**, *749*, 90–108.
16. Schwyzer, R. *Naturwissenschaften* **1966**, *53*, 189–197.
17. Bergmann, M.; Zervas, L. *Ber. Dtsch Chem. Ges.* **1932**, *65*, 1192–1201.
18. Klemm, P. In *Methods in Molecular Biology*; Walker, I. M., Ed.; Humana Press: Clifton, New Jersey, 1984; pp 243–254.
19. Carpino, L.; Han, G. *J. Org. Chem.* **1972**, *37*, 3404–3409.
20. Baillie, A. J.; Coombs, G. H.; Dolan, T. F.; Hunter, C. A.; Laakso, T.; Sjöholm, I.; Stjärnkvist, P. *J. Pharm. Pharmacol.* **1987**, *39*, 832–835.
21. Trouet, A.; Masquelier, M.; Baurain, R.; Deprez-De Campeneere, D. *Proc. Natl. Acad. Sci.* U.S.A. **1982**, *79*, 626–629.
22. Duncan, R.; Cable, H. C.; Lloyd, J. B.; Rejmanova, P.; Kopeček, J. *Bioscience Rep.* **1982**, *2*, 1041–1046.
23. Rich, D.; Singh, S. In *The Peptides*; Gross, E., Meienhofer, I., Eds.; Academic Press: New York, 1979; Vol. 1, pp 242–261.
24. Weygand, F.; Hoffmann, D.; Prox, A. Z. *Naturforsch. Teil B*, **1968**, *23*, 279–281.

Acknowledgments

The authors thank Drs. Ulf Ragnarsson and Leif Grehn for the fruitful discussions, and Ms Lena Degling for the work done on the EACA-peptides. We also thank Mrs Åsa Bjurling and Jane Timbäck for the skillful technical assistance. This work was financially supported by the Swedish Medical Research Council (Project 03X-7138) and the I. F. Foundation for Pharmaceutical Research.