Synthesis and *in Vitro* Evaluation of Potential Antichagasic Dipeptide Prodrugs of Primaquine

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Abstract □ American trypanosomiasis (Chagas' disease) is an endemic parasitic disease afflicting more than 20 million people in Latin America. Currently, therapy is unsatisfactory and only two drugs are available. Primaquine, an antimalarial drug, has trypanocidal activity. Dipeptide derivatives of primaquine, Phe-Arg-PQ, Lys-Arg-PQ, and Phe-Ala-PQ, were synthesized. The choice of the peptides was based on the primary specificity of cruzipain, the major cysteine proteinase from *T. cruzi*. The prodrugs obtained were tested on the LLC-MK_2 cell culture infected with trypomastigotes forms of *T. cruzi*. Phe-Arg-PQ, Lys-Arg-PQ, and Phe-Ala-PQ were active in all stages.

Introduction

Chagas' disease represents a serious health problem in at least 17 countries of the American continent,[1−4] where ~100 million live at the risk of contracting the disease and 16 to 18 million are indeed infected.[5] Because of the progressive rural-urban migration process and the increasing number of blood transfusions, transmission by this route has gained importance.[6]

Current therapy is unsatisfactorily accomplished with the only two available trypanocidal drugs, nifurtimox and benznidazol. These drugs are effective in the acute phase of the disease, however, their use is hampered because early diagnosis of the parasitosis is difficult and the drugs have serious adverse side effects.[3] Nevertheless, patients with Chagas' disease in Brazil are treated with benznidazol, the only drug available in the market, in spite of the claim that Brazilian *T. cruzi* strains are more resistant to this drug than strains from other countries in South America.[7] Thus, new and better drugs are urgently needed to face this serious situation.

Primaquine (PQ), which has achieved major therapeutic application as an antimalarial drug, has also been used in human acute and congenital cases of Chagas' disease.[8,9] Although the mechanism is not completely understood, it has been proposed that PQ acts as a trypanocidal agent through redox cycling with generation of active oxygen species.[10] The use of PQ, however, is limited by its toxic effects, among them hemolytic anemia, particularly in patients who are deficient in glucose-6-phosphate dehydrogenase.

Molecular modification[3] has been a most promising approach to introduce new drugs in therapeutics. Latentiation,[3,11−13] which is defined as the transformation of a drug to an inactive transport form that once biotransformed, by means of chemical or enzymatic modification, releases the drug at the site of action or near it, is commonly employed. Some derivatives obtained through this process are called prodrugs. Because some drugs that are directly linked to the carrier can not be always fully released by lysosomal enzymes,[14,15] suitable spacer groups have been developed to eliminate this drawback.[15]

Amino acids and peptides have been frequently employed as carriers because the resulting prodrugs have their bioavailabilities increased by the relative increase of hydrosolubility and/or reduction of toxicity that are generally associated to these carriers.[16] This approach emerged from the work of Carl *et al.*[17] in an attempt to diminish the toxicity of antineoplasic drugs. Later, many other drugs, such as tetracyclin,[18] hydrocortisone,[19] oxazepam and lorazepam,[20] methronidazole,[21] phenytoin,[22] and 5-aminosalicylic acid,[23] were also latentiated by their transformation to amino acid or peptide derivatives. Trouet and co-workers[15,24] have shown that amino acid and peptide derivatives of PQ with antimalarial activity were less toxic than unmodified PQ. The peptides employed were selectively cleaved by plasmin, an approach first used by Chakravarty *et al.*[25] Further, Duncan *et al.*[26] demonstrated that antineoplasic agents, such as daunomycin and doxorubicin, were released from water-soluble *N*-(2-hydroxypropyl)metacrylamide (HPMA) copolymers when linked to the polymer *via* different oligopeptides. They observed that endopeptidases first cleaved the spacer group and that, subsequently, the drug was released by means of aminopeptidases.

Comparative biochemical studies between parasites and hosts have been an important tool for chemotherapeutic drug design.[27] In addition to drug-targeting, the design of compounds that selectively inhibit enzymes necessary for the parasite survival within the host is a potent strategy for the treatment of diseases caused by parasites.[11,28] Specific targets for parasite elimination were exploited by this approach,[29,30] including specific parasite proteases, which have attracted considerable attention during the last five years. It is now recognized that in addition to their role in general protein turnover, these proteinases [cruzipain (or cruzain) being the major *T. cruzi* protease] are crucially involved in many aspects of host−parasite interaction.[31] Cruzipain was isolated, characterized, and shown to catalyze the hydrolysis of peptide bonds with arginine and lysine at the P_1 position.[31,32] Recently, Harth *et al.*[33] studied the inhibitory effect of two fluormethylketones (FMK), derivatives Z-Phe-Arg-FMK and Z-Phe-Ala-FMK, on cruzipain and concluded that the first was more potent than the last or even than FMK itself. These authors also observed that the trypanocidal effect of Z-Phe-Ala-FMK on LLC-MK_2 cultured cells infected with trypomastigote forms of *T. cruzi* is superior to that of Z-Phe-Arg-FMK.

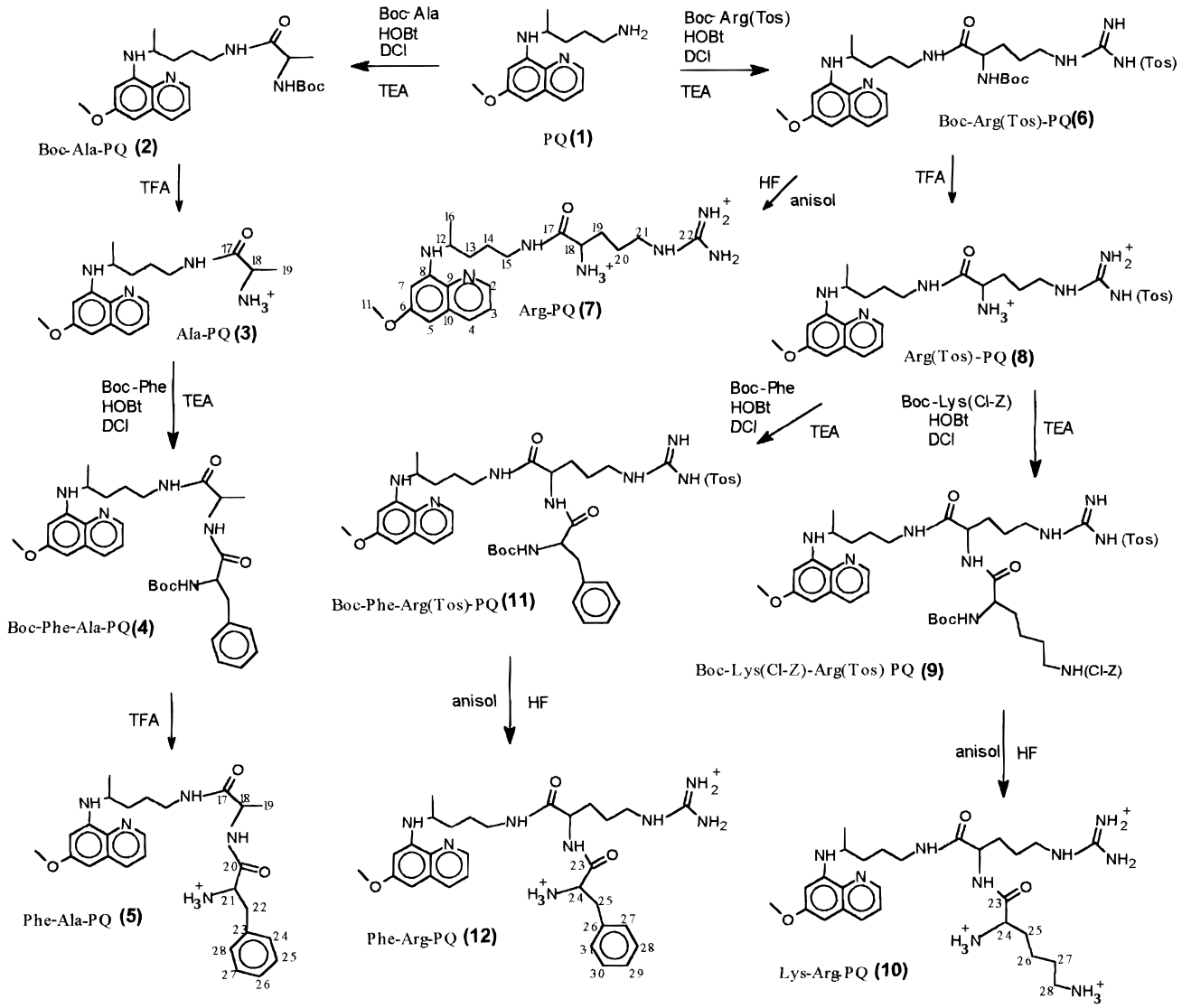
In this paper, the design, synthesis, purification, characterization, and *in vitro* trypanocidal activity of the following PQ prodrugs is reported. Arg-, Ala-, Phe-Arg-, Phe-Ala-, and Lys-Arg-PQ.

Materials and Methods

Materials—1-Hydroxybenzotriazole (HOBt), *N*,*N*′-diisopropylcarbodiimide (DIC), and trifluoroacetic acid (TFA) were from Sigma

⊗ Abstract published in *Advance ACS Abstracts*, August 1, 1997.

S0022-3549(97)00006-3 CCC: $14.00



Scheme 1—Synthesis of the aminoacyl- and dipeptide PQ derivatives: synthetic routes and chemical structures of the obtained compounds.

Chemical Company, and *N*-methylmorpholine (NMM), and triethylamine (Et_3N) were from Merck. Primaquine, [8-(4-amino-1-methylbutylamino)]-6-methoxyquinoline (PQ), as the diphosphate salt, was from FURP (Fundação do Remédio Popular, São Paulo, Brazil). N^a-*tert*-butyloxycarbonyl)-N^g-*p*-toluenesulfonyl-L-arginine [Boc-Arg(Tos)-OH], N^a-*tert*-butyloxycarbonyl-L-phenylalanine (Boc-Phe-OH), and N^a-tert-butyloxycarbonyl-L-alanine (Boc-Ala-OH) were from Protein Research Foundation, Osaka, Japan, and N^a-*tert*-butyloxycarbonyl-N^e-benzyloxycarbonyl-L-lysine [Boc-Lys(Cl-Z)-OH] was from Applied Biosystem, Inc. All other reagents (solvents and salts) of analytical grade and acetonitrile (ACN) of HPLC grade were from Merck.

Apparatus—The ^1H and ^{13}C NMR spectrometry was carried out on a NMR spectrometer AC-200, 200 MHz, in Centrais Analíticas-IQ, USP and UNESP-Araraquara, Brazil. The solvents used were $CHCl_3$-d_6 and DMSO-d_6. Mass spectra were obtained on Finnigan MAT Ltd. and Hewlett Packard (HP 5988A) spectrometers in Central Analítica-IQ-USP-São Paulo, Brazil. Melting points were performed with a Mel Temp II apparatus and are uncorrected. Amino acid analyses were performed using a Beckmann high-performance 7300 amino acid analyser adapted with a Waters 745 B integrator. Peptide hydrolyses were carried out in HCl (6 N) and phenol for 24 h at 110 °C on a Waters "Pico-Tag" WorkStation. Thin-layer chromatography (TLC) plates of silica-gel 60 F_{254} (0.25 mm thick) from Merck were used. The mobile phases were (A) chloroform:methanol:acetic acid (85:10:5; v/v/v) and (B) butanol:water:acetic acid (4:1:1; v/v/v). Reversed-phase high-performance liquid chromatography (RP-HPLC) analyses were performed on a LDC chromatograph consisting of two pumps (ConstaMetric 3200) and a Lambda Max 481 detector. The purification was carried out with a C-18 Beckman column (5 μm, Ultrasphere, 300 A, 10 mm × 25 cm) with the following mobile phases: Solvent A, water:acetonitrile (ACN):TFA (99:1:0.1, v/v/v); Solvent B, ACN:water:TFA (60:40:0.09, v/v/v). The flow rate was 3 mL/min and λ = 220 nm. The following gradients were used: Arg-PQ = 20−50% B, 30 min; Lys-Arg-PQ = 20−50% B, 30 min; Phe-Arg-PQ = 40−70% B, 30 min; Ala-PQ = 35−65% B, 35 min; and Phe-Ala-PQ = 35−65% B, 35 min.

The analyses were carried out with a C-18 Vydac column (5 μm, 300 A, 0.46 × 25 cm). The experimental conditions were: λ = 220 nm, flow rate = 1 mL/min, mobile phases = TFA [Solvent A, water:TFA (100:0.1, v/v), Solvent B, ACN:water (60:40:0.09; v/v/v)] or phosphate-triethylamine buffer at pH 2.05 (TEAP); [Solvent A, TEAP (100); Solvent B, acetonitrile:TEAP (60:40 v/v)]. The following gradients were used: Arg-PQ = 20−50% B (TFA), 30 min; 5−35% B (TEAP), 30 min; Lys-Arg-PQ = 20−50% B (TFA), 30 min; 5−35% B (TEAP), 30 min; Phe-Arg-PQ = 40−70% B (TFA), 30 min; 30-60% B (TEAP), 30 min; Ala-PQ = 35−65% B (TFA), 35 min; 30−60% B (TEAP), 30 min; Phe-Ala-PQ = 35−65% B (TFA), 35 min; 30−60%B (TEAP), 30 min.

General Synthetic Methods—The syntheses were carried out by classical chemical peptide synthesis methods.[34,35] The procedure used to prepare the aminoacyl and dipeptide PQ derivatives is shown in Scheme 1. Primaquine free base (**1**) was coupled with Boc-Ala-OH using DIC and HOBt (1.2 Boc-aa:1.2 DIC:1.2 HOBt:1.0 PQ) to give the protected alanyl-PQ derivative **2** (Boc-Ala-PQ). Removal of the Boc group of **2** produced the free aminoacyl PQ derivative Ala-PQ.TFA (**3**). This compound was reacted with Boc-Phe-OH to obtain Boc-Phe-

Ala-PQ (**4**). Removal of Boc group of the derivative **4** gave the desired Phe-Ala-PQ.TFA (**5**). Compound **6** [Boc-Arg(Tos)-PQ] was obtained by coupling PQ diphosphate with Boc-Arg(Tos)-OH. Treatment of **6** with HF gave Arg-PQ.HF (**7**). The derivative **6** was Boc deprotected to give **8** [Arg(Tos)-PQ.TFA]. Boc-Lys(Cl-Z)-Arg(Tos)-PQ (**9**) was obtained through the coupling of **8** with Boc-Lys(Cl-Z)-OH. HF treatment of **9** yielded Lys-Arg-PQ (**10**). The coupling of the derivative **8** to Boc-Phe-OH gave Boc-Phe-Arg(Tos)-PQ (**11**), which was fully deprotected using HF to yield the dipeptide derivative Phe-Arg-PQ.HF (**12**). All the reactions were monitored by TLC with appropriate solvent systems. The deprotected aminoacyl and dipeptide PQ derivatives were purified by RP-HPLC. The final compounds were characterized by RP-HPLC, ^{1}H and ^{13}C NMR, mass spectrometry and amino acid analyses.

Syntheses—*Boc-Ala-PQ (2)*—One millimole of PQ diphosphate (**1**) was dissolved in dimethylformamide (DMF) and triethylamine (TEA) in an acetone-dried ice bath. Boc-Ala-OH (1.2 mmol) was added. The pH of the solution was adjusted to 7−8 with *N*-methylmorpholine (NMM). HOBt (1.2 mmol) and DIC (1.2 mmol) were added and the pH was again adjusted to 7−8. The reaction mixture was stirred overnight at room temperature and monitored by TLC (chloroform: methanol:acetic acid, 95:5:3, v/v/v). The mixture was diluted with 0.2 M Na_2CO_3, (pH 11), and the product was extracted with ethyl ether and washed with 0.2 M citric acid solution (pH 2). After drying over Na_2SO_4, the solvents were eliminated and the crude compound was dissolved in water and lyophilized.

Ala-PQ (3)—Removal of the Boc group was accomplished with TFA 50% in methylene chloride. The reaction was monitored by TLC (chloroform:methanol:acetic acid, 95:5:3, v/v/v). After total deprotection, the solvents were eliminated and the PQ derivative was dissolved in water and lyophilized: yield, 59% (oil); R_f (A) = 0.47; R_f (B) = 0.46; mass spectrometry: M^+ = 330.2, M^{+1} =331.2, *m/e* (intensity,%) = 70 (6.34), 159 (9.8), 201 (100), 259.2 (3.5), 287.1 (3.83); ^{1}H NMR (DMSO-d_6): 8.54−8.51 (dd, 1H, Het-H), 8.36 (m, 1H, NHCO), 8.09−8.05 (d, 1H, Het-H), 7.45−7.39 (q, 1H, Het-H), 6.48−6.46 (d, 1H, Het-H), 6.26 (s, 1H, Het- H), 3.81 (s, 3H, OCH_3), 3.13 (ml, 3H, CH-*CH*$_3$), 2.87 (s, 2H,*CH*$_2$-NH_2), 1.53 (ml, 4H, CH_2-CH_2), 1.32−1.27 (dd, 3H, $C_{(17)}H_3$), and 1.21−1.18 (d, 6H, $C_{(16)}H_3$); ^{13}C NMR (DMSO-d_6, ppm): 169.19 (C_{17}), 159.03 (C_6), 144.50 (C_8), 144.20 (C_2), 135.01 (C_9), 134.37 (C_{10}), 129.65 (C_4), 122.13 (C_3), 96.34 (C_5), 91.78 (C_7), 54.99 (C_{11}), 48.21 (C_{18}), 46.91 (C_{15}, C_{12}), 33.19 (C_{13}), 25.70 (C_{14}), 20.18 (C_{16}) and 17.18 (C_{19}); amino acid analysis: alanine was detected; RP-HPLC purity, 99%.

Boc-Phe-Ala-PQ (4)—Derivative **4** was obtained by reacting Boc-Phe-OH (1.0 mmol) with **3** (1.2 mmol) according to the method used in the synthesis of derivative **2**. The deprotection was accomplished to obtain Phe-Ala-PQ (**5**): yield, 52% (oil); R_f (A) = 0.59; R_f (B) = 0.56; mass spectrometry: M^+ = 477.4, M^{+1} = 478.4, *m/e* (intensity, %) = 91 (22.82), 201(100), 259.2 (3.64), 330.3 (0.62), 462.3 (0.70); ^{1}H NMR (DMSO-d_6): 8.52−8.50 (dd, 1H, Het-H), 8.11 (ml, 1H. NH), 8.09−8.08 (d, 1H, Het-H), 8.048−8.40 (d, 1H, Het-H), 7.44−7.42 (q, 1H, 1H, Het-H), 7.22 (m, 5H, Ar-H), 6.47−6.45 (d, 1H, Het-H), 6.25 (d, 1H, Het-H), 3,80 (s, 3H, OCH_3), 3.54 (m, *CH*-CH_3, H_2O), 3.04 (m, 2H, *CH*$_2$-NH_2), 1.53−1.45 (m, 4H, CH_2-CH_2), and 1.20−1.17 (d, 6H, CH-*CH*$_3$); ^{13}C NMR (DMSO-d_6, ppm): 171.16 (C_{20}), 167.41 (C_{17}), 159.00 (C_6), 144.60 (C_8), 111.20 (C_2), 134.81 (C_9, C_{10}), 129.59 (C_4), 129.45 (C_{25}, C_{27}), 128.45 (C_{24}, C_{28}), 127.11 (C_{23}), 122.11 (C_3), 96.16 (C_5), 91.65 (C_7), 55.14 (C_{21}), 54.98 (C_{11}), 48.34 (C_{18}), 46.97 (C_{15}, C_{12}), 33.34 (C_{13}), 25.90 (C_{14}), 20.21 (C_{16}), and 18.62 (C_{19}); amino acid analysis, Phe (1.00), Ala (1.15); RP-HPLC purity, 99%.

Boc-Arg(Tos)-PQ (6)—PQ (**1**) (1.0 mmol) was dissolved in DMF and TEA in an acetone-dried ice bath. Boc-Arg(Tos)-OH (1.2 mmol) was then added and the pH of the solution was adjusted to 7−8. Next, HOBt (1.2 mmol) and DIC (1.2 mmol) were added and the pH was again adjusted to 7−8 with NMM. The reaction was carried out overnight at room temperature and monitored by TLC (chloroform: methanol:acetic acid, 95:5:3, v/v/v). Then, 0.2 M Na_2CO_3 solution was added to the reaction mixture and the product was extracted with ethyl ether and washed with 0.2 M citric acid solution. After drying over Na_2SO_4, the solvents were eliminated and the crude compound was dissolved in water and lyophilized.

Arg-PQ (7)—Removal of the protective groups Boc and Tos was carried out by HF treatment in 5% anisol for 1.5 h. The acid excess and the anisol were eliminated with high vacuum. The resulting residue was dissolved in water and lyophilized. The purification was performed by RP-HPLC and the purified compound was characterized: yield, 72%; mp, 162−164 °C; R_f (A) = 0.49; R_f (B) = 0.48; mass spectrometry: M^+ = 414.2, M^{+1} = 415.2, *m/e* (intensity): 59 (17.2), 70 (100), 87 (56.98), 175 (19.35), 201 (53.76), 259 (8.38); ^{1}H NMR (DMSO-d_6, d): 8.53−8.51 (d, 1H, Het-H), 8.46 (ml, 1H, NH), 8.16 (ml, 2H, NH_2), 8.09−8.05 (d, 1H, Het-H), 8.84 (ml, 1H, NH), 7.84 (ml, 1H, NH), 7.45−7.38 (q, 1H, Het-H), 6.47 (d, 1H, Het-H), 6.26 (d, 1H, Het-H), 3.80(s, 3H, OCH_3), 3.67 (m, 1H, *CH*CH_3), 3.14−3.10 (ml, 2H, *CH*$_2$-NH_2), 1.64−1.53(ml, CH_2), and 1.20−1.17 (d, 3H, CH_3); ^{13}C NMR (DMSO-d_6, ppm): 168.06 (C_{17}), 156.86 (C_{22}), 158.98 (C_6), 144.57 (C_8), 144.25 (C_2), 134.89 (C_9), 129.61 (C_4), 122.14 (C_3), 96.19 (C_5), 91.78 (C_7), 54.98 (C_{11}), 51.96 (C_{18}), 46.95 (C_{15}, C_{12}), 33.41 (C_{13}), 15.78 (C_{20}), 24.23 (C_{14}), and 20.20 (C_{16}); amino acid analysis: arginine was detected; RP-HPLC purity, 97%.

Arg(Tos)-PQ.TFA (8)—The removal of the Boc group was accomplished with 50% TFA in methylene chloride. The reaction was monitored by TLC (chloroform:methanol:acetic acid, 95:5:3, v/v/v). After total deprotection, the solvents were eliminated and the PQ derivative was dissolved in water and lyophilized.

Boc-Lys(Cl-Z)-Arg(Tos)-PQ (9)—Coupling of Boc-Lys(Cl-Z)-OH with **8** produced **9**. Isolation of **9** from the reaction medium was accomplished as described for **6**.

Lys-Arg-PQ (10)—Deblocking was proceeded by treatment of **9** with HF/anisol (5%) for 1.5 h. HF and anisol were eliminated with high vacuum. The product was dissolved in water, lyophilized, and purified: yield, 86%; mp, 100−102 °C; R_f (A) = 0.13; R_f (B) = 0.11; mass spectrometry: *m/e* (intensity, %) = 59.1; 84.9 (100); 123 (74.18); 155 (60.15); 232 (54.96); 259.2(18.61); 303.1 (19.36); 356.1 (46.05); 414.2 (5.45); ^{1}H NMR (DMSO-d_6): 8.52−8.50 (dd, 1H, Het-H); 8.08−8.05 (dd, 1H, Het-H), 7.70−7.67 (ml, 1H, NH), 7.60−7.67(ml, 1H, NH), 7.44−7.38 (q, 1H, Het-H), 6.47−6.46 (d, 1H, Het-H), 6.24 (m, 1H, Het-H), 3.80 (s, 3H, OCH_3), 3.34 (m, H_2O), 2.79−2.72(t, *CH*-NH_2), 1.53−1.42 (m, 1H, NH), and 1.19−1.16 (d, 3H, CH_3); ^{13}C NMR (DMSO-d_6, ppm); 170.08 (C_{17}), 158.97 (C_6), 156.70 (C_{22}), 144.59 (C_8), 144.24 (C_2), 134.80 (C_9), 134.53 (C_{10}), 129.80 (C_4), 122.11 (C_3), 96.24 (C_5), 91.78 (C_7), 54.97 (C_{11}), 46.95 (C_{15}, C_{12}), 33.38 (C_{13}), 26.85 (C_{20}), and 20.18 (C_{16}); amino acid analysis: Lys (0.96), Arg (1.00); RP-HPLC purity, 90%.

Boc-Phe-Arg(Tos)-PQ (11)—This derivative was obtained by coupling Boc-Phe-OH (1.2 mmol) with **8** (1.0 mmol) in DMF and TEA in an acetone-dried ice bath. The pH of the solution was adjusted to 7−8, and HOBt (1.2 mmol) and DIC (1.2 mmol) were added. The pH was corrected to 7−8 with NMM. The reaction was carried out overnight at room temperature and monitored by TLC (chloroform: methanol:acetic acid, 95:5:3, v/v/v). Then, 0.2 M Na_2CO_3 solution was added to the reaction mixture and the product was extracted with ethyl ether and washed with 0.2 M citric acid solution. After being dried over anhydrous Na_2SO_4, the organic layer was filtered and evaporated.

Phe-Arg-PQ (12)—After 2 deblocking reaction with HF/5% anisol for 1.5 h, the deprotected product was lyophilized and submitted to RP-HPLC purification and analysis: yield, 60,1%; mp, 158−160 °C (dec.); R_f (A) = 0.58; R_f (B) = 0.57; mass spectrometry: *m/e* (intensity, %) = 84.9 (12.95), 91 (31.70), 120 (22.53), 128 (4.46), 201 (100), 259.2 (18.00), 303.1 (3.52), 470.3 (0.51); ^{1}H NMR (DMSO-d_6): 8.52−8.50 (dd, 1H, Het-H), 8.08−8.04 (dd, 1H, Het-H), 7.98 (ml, 1H, NH), 7.65 (ml, 1H, NH), 7.44−7.38 (q, 1H, Het-H), 7.21−7.19 (m, 5H, Ar-H), 6.46−6.45 (d, 1H, Het-H), 6.25−6.24 (d, 1H, Het-H), 6.12−6.08 (d, 1H, NH), 3.80 (s, 3H, OCH_3), 1.53−1.45 (m, CH_2), and 1.19−1.16 (d, 3H, CH_3); ^{13}C NMR (DMSO-d_6, ppm): 170.70 (C_{17}), 158.97 (C_6), 156.70 (C_{22}), 144.59 (C_8), 144.24 (C_2), 134.81 (C_9), 134.52 (C_{10}), 129.57 (C_4), 129.32 (C_{28}, C_{30}), 128.16 (C_{27}, C_{31}), 126.35 (C_{26}), 122.11 (C_3), 96.11 (C_5), 91.61 (C_7), 54.97 (C_{11}), 46.94 (C_{12}, C_{15}), 33.38 (C_{13}), 29.05 (C_{19}), 25.88 (C_{20}), 24.95 (C_{14}) 20.19 (C_{16}), and 18.62 (C_{19}); amino acid analysis, Phe (1.11), Arg (1.00); RP-HPLC purity, 99%.

Biological *In Vitro* Tests—*Trypanosomes*—Trypomastigote forms of *Trypanosoma cruzi*, Y strain, were used in the *in vitro* assays. The organism was maintained in continuous culture in Dulbecco modified Eagle medium[36] supplemented with $NaHCO_3$ (1.2 g/L), penicillin (500 000 U/L) and streptomycin (100 mg/L) (DME medium), containing 10% of heat-inactivated fetal bovine serum at 37 °C.

Cell Culture Assays—The *in vitro* testing was performed by seeding 24/2 mL wells tissue culture slides with 2×10^4 Rhesus monkey kidney epithelial cells (*Macaca mullata*), LLC-MK_2 (provided by Instituto Adolfo Lutz, São Paulo, Brazil) maintained in DME supplemented with 5% heat-inactivated fetal bovine serum. Slides were incubated at 37 °C in an atmosphere containing 5% CO_2 in air.

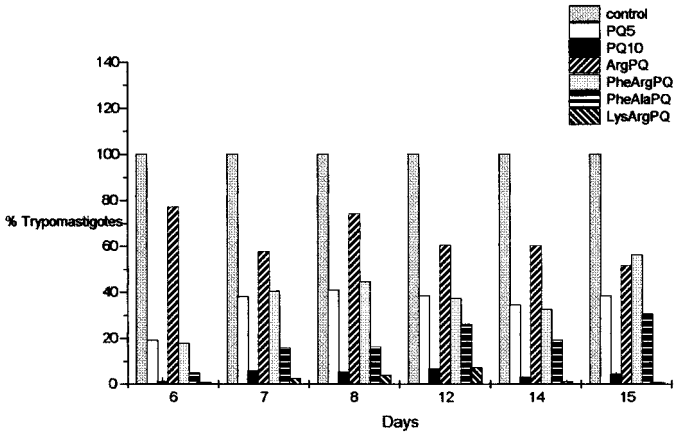


Figure 1—Effect of PQ5, PQ10, Arg-PQ, Phe-Arg-PQ, Phe-Ala-PQ, and Lys-Arg-PQ on the percentage of trypomastigotes at 6, 7, 8, 12, 14 and 15th days after cell infection with *T. cruzi* trypomastigotes in comparison with the control (PQ5 = 5 μM; PQ10 = 10 μM; the other compounds were tested at a concentration of 10 μM).

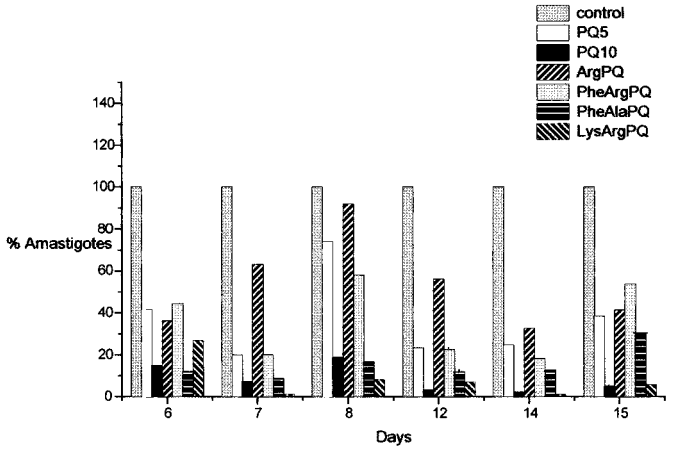


Figure 2—Effect of PQ5, PQ10, Arg-PQ, Phe-Arg-PQ, Phe-Ala-PQ, and Lys-Arg-PQ on the percentage of amastigotes at 6, 7, 8, 12, 14 and 15th days after cell infection with *T. cruzi* trypomastigotes in comparison with the control.

Confluent cells were used for subcultivation. The cell monolayer was washed with 10 mM phosphate buffer-150 mM NaCl (PBS) and treated with 0.1% trypsin in PBS containing 1 mM of EDTA. The subcultivation was repeated every 7 days. Drugs were dissolved in DMSO or water and diluted to the desired concentrations with the medium. Final solvent concentrations did not exceed 0.1% (v/v). Trypomastigotes (1×10^6/mL) were inoculated 24 h after the cells layers were seeded. Medium containing test drugs in concentrations of 5 and 10 μM (final concentration) were added to the cells. After incubating for 24 h, the media were changed daily by new ones (containing or not containing drugs). The *in vitro* activity was determined by counting the number of organisms at 6, 7, 8, 12, 14, and 15 days after infection. The organisms were counted separately as trypomastigotes and amastigotes.

Results and Discussion

Peptide prodrugs of PQ were synthesized based on a putative specificity to cruzipain. Amino acids and peptides, such as Arg, Phe-Arg, Phe-Ala, and Lys-Arg, were used as carriers, and syntheses were performed with HOBt (1-hydroxybenzotriazole) and DIC (diisopropylcarbodiimide) as coupling agents. Product analyses confirmed that the planned compounds were obtained. In general, the syntheses were particularly difficult because of the presence of PQ, which is not chemically very stable under the experimental conditions used. The formation of some unexpected byproducts was observed in most, but not all of, the cases. These observations are in accordance with those of Borissova *et al.*,[16] who reported difficulties during several syntheses because of PQ photosensitivity and alkaline instability, and those of Kristensen *et al.*[37] confirming the photochemical degradation of that drug. Because of this photosensitivity, the reactions were protected from light, especially those performed in aqueous media. Most of the reports dealing with peptide derivatives of PQ[14,16,38,39] used DCC as the coupling agent. Only the synthesis of PQ derivatives starting from *N*-(tert-butyloxycarbonyl)-ϵ-aminocaproic acid used DIC without the need of maintaining the pH to 7−9 during the reaction.[16] In this work, however, the success in obtaining Ala-PQ, Arg-PQ, Phe-Ala-PQ, Phe-Arg-PQ, and Lys-Arg-PQ in reasonable yields was fully dependent on maintenance of the pH at 7−9.

The biological tests were conducted with LLC-MK_2 cell cultures infected with trypomastigote forms of *T. cruzi*. The activity of PQ and its derivatives in trypomastigote development after 6, 7, 8, 12, 14, and 15 days was followed (Figure 1). Phe-Arg-PQ, Phe-Ala-PQ, and Lys-Arg-PQ, as well as 5 and 10 μM PQ (PQ5 and PQ10, respectively) significantly inhibited trypomastigote development compared with the control without drug. Arg-PQ was much less active than the other derivatives. The fact that the inhibition profile is the same from the 6th to the 15th day of culture shows that infection was not fully aborted and delivery of trypomastigotes to the culture medium, although highly diminished, was not affected by the presence of the drugs. An overall apraisal of the inhibition kinetics shows that Phe-Arg-PQ and Arg-PQ are not significantly different in their partial inhibitory capacities. Phe-Ala-PQ shows more appreciable activity, although it becomes relatively less efficient as the experiment proceeds. Interestingly, these findings are in accordance with those by Harth *et al.*[33] who sugested that Phe-Ala-FMK was a better inhibitor of *T. cruzi* development in cell cultures than Phe-Arg-FMK because of permeability differences of both compounds. The addition of a lysine to the compound Arg-PQ rendered a highly effective inhibitor of trypomastigote development that could be due to a higher capacity of Lys-Arg-PQ to be absorbed by the cells and to the fitness of this substrate to *T. cruzi* cruzipain.

The effect of PQ and aminoacyl-PQ on the number of intracellular amastigotes follows roughly the pattern described for trypomastigotes (Figure 2). This result indicates that inhibition of development occurs early in the transformation of trypomastigotes to amastigotes, thus implicating proteolysis as involved in this differentiation step.

In summary, Lys-Arg-PQ is the most active of the modified compounds tested, with effects similar to 10 μM PQ. However, considering the peptide derivative is less toxic than PQ, it would be of interest to test the derivative *in vivo* with experimental animals. Additional studies on absorption and cell toxicity of this compound are being undertaken and will be published elsewhere.

Conclusions—Knowledge of parasite proteases is extremely important to find potential targets for drug design. With this objective, we synthesized four aminoacyl derivatives of PQ that could be selectively cleaved by cruzipain, a specific protease of *T. cruzi*. Our observations support the conclusion that Lys-Arg-PQ is active on *T. cruzi* development inside host cells, probably by interfering in the initial steps of trypomastigote-amastigote transformation. The finding that Lys-Arg-PQ is more active than Phe-Ala-PQ and Phe-Arg-PQ suggests that the specific cleavage has an important role in the release of PQ. The dipeptide Lys-Arg, putatively specific for cruzipain, was a good carrier for PQ and has potential to be used as a spacer group for the development of other PQ prodrugs in the future. Thus, Lys-Arg-PQ is a very promising compound for *in vivo* tests.

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Acknowledgments

This work was supported by grants from FAPESP (Project # 93-3547-5) to M. T. M. Miranda, from FINEP/FNDCT (Project # 66.950.825-00) to E. I. Ferreira, from CAPES/PICD to M. C. Chung, and from PADCT/CNPq (Project # 62.0251/94-8) and FAPESP (Thematic Project # 95/4562-3) to W. Colli. The authors acknowledge I. N. Toma for the amino acid analyses, and N. Boralli and M. I. A. Gonçalves for the NMR analyses and interpretation.

JS970006V