Int. J. Peptide Protein Res. 29, 1987, 262–275

Synthesis and biological activity of tuftsin and rigin derivatives containing monosaccharides or monosaccharide derivatives

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Received 29 May, accepted for publication 23 July 1986

Synthesis of some modified rigins is described in which either D-gluconic acid or 2-amino-2-deoxy-β-D-glucopyranose have been linked to the parent molecule through amide bonds involving the α-amino function, α-carboxyl function or the γ-amide function of glutamine in position 2. Glu[2]-rigin and D-gluconyl-Glu[2]-rigin have also been synthesized. Binding and phagocytosis assays have been carried out on the rigin derivatives and on some glycosylated tuftsin derivatives as well. Of all the tested peptides only rigin enhanced the phagocytic capacity of mouse peritoneal macrophages to the same extent as tuftsin. The peptides H-Thr-Lys-Pro-Arg-NH-Glc and N^{α}-gluconyl-Gly-Glu-Pro-Arg-OH slightly enhanced phagocytosis. H-Thr[(α + β)-*O*-glucosyl]-Lys-Pro-Arg-OH was found to displace ^{3}H-tuftsin even better than tuftsin but lacked the ability to stimulate phagocytosis.

Key words: binding assays; glycosylated rigins; peptide synthesis in solution; phagocytosis; pseudoglycopeptides; rigin

In the preceding paper (1) we reported the preparation of some modified tuftsins containing a monosaccharide or a monosaccharide derivative covalently linked to the parent molecule. In particular, syntheses have been described of N^{α}-D-gluconyl-tuftsin, 2-deoxy-2-tuftsinamido-D-glucopyranose and *O*-glucosyl-tuftsin.

It has been demonstrated (2) that the phagocytosis stimulating activity of the synthetic tetrapeptide rigin, Gly-Gln-Pro-Arg, corresponding to the 341–344 amino acid sequence of IgG human H-chain is equal to that of tuftsin, Thr-Lys-Pro-Arg.

Following our work (1, 3) on the effect of covalently linking a monosaccharide, or a

Presented in part at the 18th European Peptide Symposium, Djurönäset, Sweden, June 1984, Rocchi R., Biondi, L., Filira, F., and Gobbo, M., Abstracts of Communications, p. 124.

The amino acid residues are of L-configuration. Standard abbreviations for amino acid derivatives and peptides are used according to the suggestions of the IUPAC – IUB Commission on Biochemical Nomenclature (1984) *European J. Biochem.* 138, 9–37. Other abbreviations used are: Boc, *tert.*-butyloxycarbonyl; Z, benzyloxycarbonyl; Bzl, benzyl; Me, methyl; Su, succinimidyl; TFA, trifluoroacetic acid; DMF, dimethylformamide; DCC, *N,N'*-dicylohexylcarbodiimide; H_2N-Glc, 2-amino-2-deoxy-β-D-glucopyranose; M.A., mixed anhydride procedure; HPLC, high performance liquid chromatography; t.l.c., thin-layer chromatography.

monosaccharide derivative, to a biologically active peptide we synthesized rigins in which either D-gluconic acid or 2-amino-2-deoxy-β-D-glucopyranose have been linked to the parent molecule through amide bonds involving the α-NH_2, the α-COOH, or the γ-amide function of Gln 2. Furthermore Glu^2-rigin and D-gluconyl-Glu^2-rigin have also been synthesized.

In some cases the use of alternative synthetic pathways and/or different activation procedures and protecting groups has been explored.

Protected rigin Z-Gly-Gln-Pro-Arg(NO_2)-OMe (III) has been synthesized in solution by the step-wise procedure shown in Fig. 1.

The carboxyl function of *N*-protected proline, glutamine and glycine was activated as succinimidyl ester and removal of the *tert.*-butyloxycarbonyl protecting groups achieved by treatment of the protected peptides with HCl in organic solvent. Final deblocking was accomplished by saponification of the methyl ester followed by catalytic hydrogenation.

The derivatization of the γ-amido function of the glutamine residue has been achieved as shown in Fig. 2.

Z-Glu(OBu^t)-OH (4) was coupled, by the mixed anhydride procedure, to H-Pro-Arg(NO_2)-OMe to give the protected tripeptide Z-Glu(OBu^t)-Pro-Arg(NO_2)-OMe (V). Removal of both the benzyloxycarbonyl and *tert.*-butyl ester groups by treatment with HBr in acetic acid followed by acylation with Z-Gly-OSu (5) yielded the partially protected tetrapeptide Z-Gly-Glu-Pro-Arg(NO_2)-OMe (VI) which was converted *via* the mixed anhydride to the corresponding γ-*tert.*-butyloxycarbonyl-hydrazide (VII).

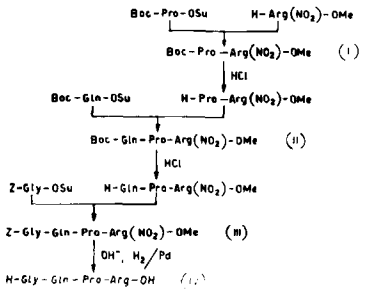

FIGURE 1
Synthesis of rigin.

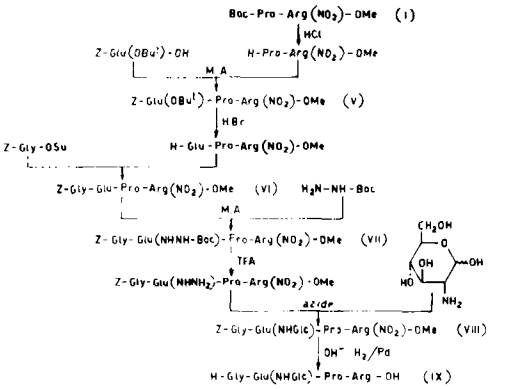

FIGURE 2
Synthesis of H-Gly-Glu(NHGlc)-Pro-Arg-OH.

Selective removal of the *tert.*-butyloxycarbonyl protecting group from Z-Gly-Glu(NHNH-Boc)-Pro-Arg(NO_2)-OMe with trifluoroacetic acid gave the corresponding γ-hydrazide, which was reacted by the azide procedure with 2-amino-2-deoxy-β-D-glucopyranose. Saponification of the resulting Z-Gly-Glu(NHGl)-Pro-Arg(NO_2)-OMe (VIII) followed by catalytic hydrogenation afforded the desired H-Gly-Glu(NHGlc)-Pro-Arg-OH (IX).

The synthetic route used in the preparation of H-Gly-Gln-Pro-Arg-NHGlc (XIII) is shown in Fig. 3. Acylation of H-Pro-OBzl (6) with Boc-Gln-OSu (7) yielded the dipeptide Boc-Gln-Pro-OBzl (X), which was selectively deprotected by trifluoroacetic acid and allowed to react with Z-Gly-OSu (5). The resulting protected tripeptide Z-Gly-Gln-Pro-OBzl (XI) was converted into the corresponding hydrazide and reacted, by the azide procedure, with H-Arg(NO_2)-NHGlc (3). The protected tetrapeptide Z-Gly-Gln-Pro-Arg(NO_2)-NHGlc (XII) was completely deblocked by catalytic hydrogenation.

An attempt at preparing compound XIII by reacting, through the azide procedure, Z-Gly-Gln-Pro-N_2H_3 with H-Arg(NO_2)-NHGlc $(OAc)_4$ followed by removal of the protecting groups, failed.

The protected tripeptide Z-Gln-Pro-Arg(NO_2)-OBzl (XV) has been prepared, through the azide

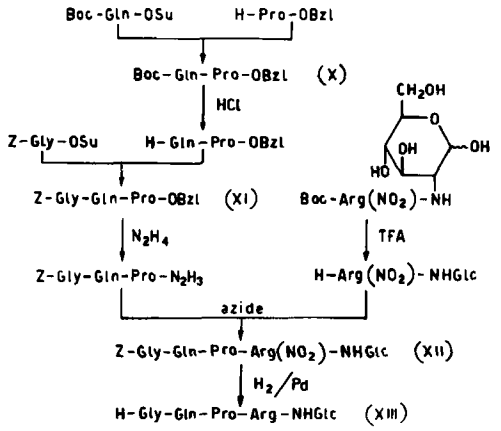

FIGURE 3
Synthesis of 2-deoxy-2-riginamido-D-glucopyranose.

procedure from Z-Gln-Pro-N_2H_3 with H-Arg(NO_2)-OBzl (8)(Fig. 4). Alternatively compound XV was obtained by acylating H-Pro-Arg(NO_2)-OBzl (9, 10) with Z-Gln-OH (11) by the mixed anhydride procedure. Selective removal of the benzyloxycarbonyl protecting group by treatment with HBr in acetic acid followed by acylation with D-gluconyl-Gly-N_2H_3 (12) yielded the D-gluconyl-Gly-Gln-Pro-Arg(NO_2)-OBzl (XVI). Catalytic hydrogenation gave the desired D-gluconyl-rigin (XVII).

Essentially the same procedure was used in the synthesis of Glu^2-rigin (XXII), and D-gluconyl-Glu^2-rigin (XXIII) (Fig. 5).

Flash chromatography either on silica gel or on LiChroprep RP-8 column has been extensively used for purification of the protected intermediates and final products. Ion exchange chromatography was used for the isolation of the fully deblocked rigin derivatives. The identity and purity of the intermediates and final products were established by elemental analysis, amino acid analysis of acid hydrolysates, optical rotation, thin-layer chromatography in several solvent systems and HPLC analysis.

EXPERIMENTAL PROCEDURES

Materials, preparative and analytical methods as well as the solvent systems and the buffers used were described in the preceding paper (1).

Macrophages

Peritoneal exudate cells were aseptically collected from thioglycolate-stimulated (Balb/c x C_3H) F1-strain female mice (6–8 weeks old). The stimulating agent, thioglycolate broth (2.98 g/100 mL, Difco Laboratories, Detroit, USA) was injected intraperitoneally (3 mL/mouse) 4 days prior to cell harvest. Cells were collected by washing the peritoneum

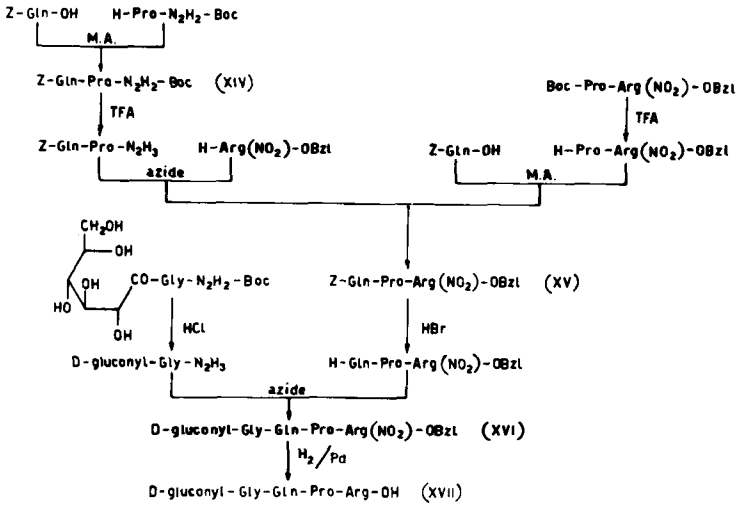

FIGURE 4
Synthesis of D-gluconyl-rigin.

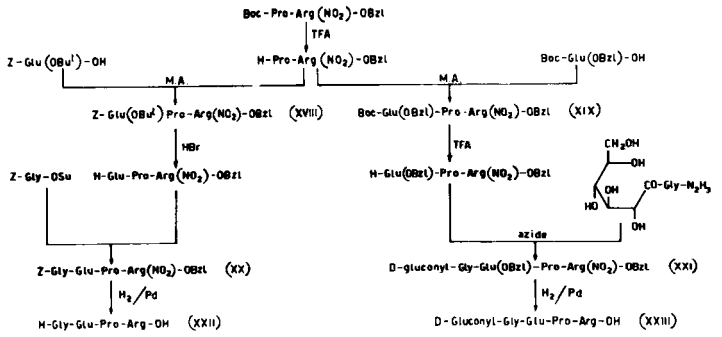

FIGURE 5
Synthesis of Glu^2-rigin and D-gluconyl-Glu^2-rigin.

with cold phosphate-buffered saline (PBS, Gibco, pH 7.4), centrifuged and resuspended in Dulbecco's modified Eagle medium (DMEM, Gibco) at a final concentration of 1.5×10^6 cells/mL. Cell preparations consistently contained >90% macrophages of which more than 87% were viable cells.

IgG-coated sheep red blood cells (SRBC)

Erythrocytes (10^9 cells suspended in 3 mL PBS) were sensitized with rabbit anti-SRBC antibodies (7-S IgG, Cortis Laboratories) (3 mL of 1:150 dilution in PBS) at 37° for 45 min. Following three washes in PBS, cells were resuspended in PBS (3 mL) and ^{51}Cr (1.5 μCi, Amersham) was added. After incubation at 37° for 2 h, cells were washed (x 3) in PBS and resuspended in PBS (30 mL).

Phagocytosis assays

Macrophages, suspended in DMEM (1.5×10^6 cells/mL) were layered on tissue culture trays (Costar, 24 wells/tray; 0.5 mL/well). After incubation at 37° for 2 h in humidified incubator (5% CO_2 in air) non-adherent cells were removed by washing (x 3) with PBS and cultures supplemented with 1 mL DMEM-5% fetal calf serum (Gibco, Grand Island, New York). Subsequent to cultivation for 24 h, cells were washed in PBS and preincubated with the tested peptides for 10 min at 37°. ^{51}Cr-IgG-coated SRBC (0.5 mL) was then added and macrophage monolayers were further incubated for 45 min at 37°. The cells were then washed once with PBS, treated with 0.83% NH_4Cl solution to remove free and surface-bound SRBC, washed again with PBS (x 3) and finally dissolved in 0.2% sodium dodecyl sulfate (SDS) (0.5 mL). The solution obtained was collected and radioactivity was measured in a Hewlett-Packard gamma counter. Results given are from triplicate wells whose SEM is less than 5%.

Competitive binding assay

All binding studies were performed for 30 min in PBS at 22° at a final volume of 0.5 mL. Macrophage was suspended in PBS (2.5×10^6 cells/mL), incubated with gentle agitation in plastic tubes (Nunc 70 x 12 mm; 1×10^6 cells/tube) with tritiated tuftsin ([^{3}H-Arg^4]-tuftsin; specific activity 20.8 Ci/mmol; 5×10^{-8} M) and varying concentrations of unlabeled tuftsin or the tested peptides. Binding was terminated by dilution with PBS (3 mL), followed by centrifugation and removal of supernatant by aspiration. Cells were subsequently washed once more with PBS and similarly isolated. The cell pellets were then dissolved in 0.3 mL SDS (0.2%) and the solutions obtained were collected and added to vials, each containing 5 mL of triton/toluene mixture. Radioactivity was measured in a Beckman 7500 liquid scintillation spectrophotometer. Each point on the resulting binding curves derives from duplicate tubes and the standard error of the mean (SEM) did not exceed 5%. Nonspecific binding was defined as the amount of tritiated tuftsin not inhibited by 10 μM tuftsin.

Peptide synthesis

Boc-Pro-Arg(NO_2)-OMe (I). Boc-Pro-OSu (5) (12.5 g, 40 mmol) was added to a solution of H-Arg(NO_2)-OMe hydrochloride (13) (12.14 g, 45 mmol) and triethylamine (6.3 mL, 45 mmol) in a mixture of H_4-furan (50 mL) and DMF (50 mL). The reaction mixture was kept 12 h at room temperature and evaporated to dryness. The residue was taken up in ethyl acetate, washed and dried, the solvent was evaporated and the oily residue was reprecipitated from ether with light petroleum (12 g, 70%); m.p. 75–83° (softening); $[\alpha]_D^{25}$ −47.2° (*c* 1.0, methanol); single spot by t.l.c. in solvent system II.

Anal. calc. for $C_{17}H_{30}N_6O_7$ (430.44): C 47.43, H 7.02, N 19.52.

Found: C 47.39, H 7.17, N 18.99.

Boc-Gln-Pro-Arg(NO_2)-OMe (II). Boc-Pro-Arg (NO_2)-OMe (5 g, 11.6 mmol) was dissolved in 1.5 N HCl in ethyl acetate (200 mL). After 60 min the resulting precipitate was collected, washed with ice-cold ethyl acetate and ether, dried *in vacuo* and dissolved in a H_4-furan/DMF mixture (200 mL, 1:1 v/v). The solution was cooled to 0°, triethylamine (3.25 mL, 23.2 mmol) and Boc-Gln-OSu (7) (3.57 g, 10.4 mmol) were added and, after 24 h stirring, the reaction mixture was evaporated to dryness. The residue was dissolved in butan-1-ol, previously saturated with water, and washed with 5% aqueous acetic acid saturated with butan-1-ol, and water saturated with butan-1-ol.

Evaporation of the solvent gave a residue which was precipitated from methylene chloride with light petroleum (4.35 g, 75%). Aliquots of the crude product (0.3 g) were purified by flash chromatography [Silica Gel F 60 column (3 × 15 cm), chloroform-methanol (95:5 v/v), flow rate 30 mL/min, fraction volume 30 mL]. Fractions showing a single spot on t.l.c. (solvent system IV) were pooled and evaporated to dryness (0.24 g, 80%); m.p. 95–97° (softening), 200–205° dec.; $[\alpha]_D^{20}$ −57.6° (*c* 1.15, methanol).

Anal. calc. for $C_{22}H_{38}N_8O_9$ (558.55): C 47.30, H 6.85, N 20.06.

Found: C 46.70, H 6.78, N 19.31.

Z-Gly-Gln-Pro-Arg(NO_2)-OMe (III). Boc-Gln-Pro-Arg(NO_2)-OMe (3.2 g, 5.7 mmol) was dissolved in a mixture of methylene chloride and ethyl acetate (10:3 v/v) (130 mL) and 4.5 N HCl in ethyl acetate (40 mL) added.

After 60 min the resulting precipitate was collected, washed with ethyl acetate, dried and dissolved in ice-cold DMF (100 mL) containing triethylamine (1.6 mL, 11.4 mmol).

Z-Gly-OSu (5) (1.57 g, 5.13 mmol) was added and the reaction mixture was stirred overnight, then evaporated to dryness. The residue was dissolved in butan-1-ol (250 mL), washed (0.5 M Na_2CO_3, 5% acetic acid, water) and dried. The solvent was evaporated, the residue dissolved in a mixture (60 mL) of methylene chloride – methanol (1:1 v/v) and precipitated with excess ether (2.83 g, 85%). Aliquots of the crude product (0.3 g) were purified by flash chromatography on a silica Gel F 60 column [3 × 15 cm, chloroform-methanol (95:5 v/v) as the eluant, flow rate 30 mL/min, fraction volume 30 mL]. Fractions containing single spot material by t.l.c. (solvent systems I, II, and III) were pooled and evaporated to dryness (0.24 g, 80%); m.p. 95–97° (softening), 122–125° dec.; $[\alpha]_D^{20}$ −43.76° (*c* 1.09, methanol).

Anal. calc. for $C_{27}H_{39}N_9O_{10}$ (649.62): C 49.92, H 6.05, N 19.41.

Found: C 49.77, H 6.14, N 19.35.

Amino acid ratios in acid hydrolysate: Pro 0.98, Glu 1.00, Gly 0.99 (Arg + Orn) 0.96.

H-Gly-Gln-Pro-Arg-OH (IV). Z-Gly-Gln-Pro-Arg (NO_2)-OMe (1.0 g, 1.54 mmol) was dissolved in a dioxane-water mixture (30 mL, 4:1 v/v) and 1 N NaOH was added (1.7 mL). After 90 min the solution was cooled in an ice-bath, acidified to pH 3 by addition of 1 N HCl and evaporated to dryness. The residue was dissolved in methanol (30 mL), diluted with 20% aqueous acetic acid (30 mL) and hydrogenated for 3 h. The catalyst was filtered off, washed with water and the solution and washings were combined, evaporated *in vacuo* and lyophilized. The residue was dissolved in 0.01 M ammonium acetate buffer (pH 5.5) and applied to a CM 52 column (2 × 35 cm) previously equilibrated with the same buffer. Elution was carried out with a linear concentration gradient of ammonium acetate prepared from 700 mL each of equilibrating

buffer and 0.4 M ammonium acetate (flow rate 36 mL/h, fraction volume 9 mL). Elution was monitored by u.v. absorption at 254 nm. The desired fractions were combined, evaporated to dryness, desalted by passage through a Sephadex G 10 column (2 x 120 cm), equilibrated and eluted with 5% aqueous acetic acid (flow rate 27 mL/h, fraction volume (6.75 mL). The product containing fractions [single spot by RP-8 t.l.c., acetonitrile-5% aqueous acetic acid (60:40 v/v) as the eluant] were combined and lyophilized (0.55 g, 67% as mono-acetate, mono-hydrate); $[\alpha]_D^{25} - 77.6°$ (*c* 1.03, 5% aqueous acetic acid). Lit. $[\alpha]_D^{22°} - 77.0°$ (*c* 0.6, 5% aqueous acetic acid)(2).

Anal. calc. for $C_{18}H_{32}N_8O_6 \cdot CH_3COOH \cdot H_2O$ (534.56): C 44.93, H 7.16, N 20.96.
Found: C 45.59, H 7.01, N 20.91.

Amino acid ratios in acid hydrolysate: Pro 1.00, Glu 1.01, Gly 0.99, Arg 0.99 (98% as mono-acetate, mono-hydrate).

Analytical RP-HPLC elution profile of the isolated rigin is shown in Fig. 6.

Z-Glu(OBu^t)-Pro-Arg(NO$_2$)-OMe (V). Boc-Pro-Arg(NO$_2$)-OMe (5.0 g, 11.6 mmol) was dissolved in 1.5 N HCl in ethyl acetate (220 mL). After 60 min the resulting precipitate was collected by filtration, dried *in vacuo*, taken up in ice-cold DMF (50 mL) and neutralized with triethylamine (3.25 mL, 23.2 mmol). The solution was added to the mixed anhydride solution prepared from Z-Glu(OBu^t)-OH (4) (3.5 g, 10.4 mmol) in DMF (50 mL) with *N*-methylmorpholine (1.16 mL, 10.4 mmol) and isobutylchloroformate (1.35 mL, 10.4 mmol) at $-15°$ for 2 min. After stirring for 10 min at $-15°$ the reaction mixture was kept overnight at room temperature and evaporated to dryness. The residue was taken up in ethyl acetate, washed and dried. Evaporation of the solvent gave an oil (6.5 g, 96%) which showed the presence of two minor contaminants by t.l.c. in solvent system IV, but was used without further purification for the next synthetic step. For the analysis an aliquot of the crude product (0.5 g) was purified by flash chromatography on the Chromatospac-Prep 100 Apparatus [90 g of Silica gel F 60, methylene chloride-methanol (98.5:1.5 v/v), flow rate 50 mL/min, fraction volume 50 mL] to give 100 mg (20%) of pure product, m.p. 64–65° (softening), $[\alpha]_D^{25} -40.6°$ (*c* 1.02, methanol), single spot by t.l.c. in solvent systems I, II, and IV.

Anal. calc. for $C_{29}H_{43}N_7O_{10}$ (649.67): C 53.61, H 6.67, N 15.09.
Found: C 53.43, H 6.81, N 14.93.

Amino acid ratios in acid hydrolysate: Pro 1.00, Glu 1.00 (Arg + Orn) 0.98.

Z-Gly-Glu-Pro-Arg(NO$_2$)-OMe (VI). Z-Glu (OBu^t)-Pro-Arg(NO$_2$)-OMe (6.0 g, 9.23 mmol) was dissolved in 33% HBr in acetic acid (18 mL). After 15 min excess ether was added and the precipitated oil collected, triturated with ether, dissolved in ethyl acetate, and reprecipitated with light petroleum. The resulting hygroscopic product (4.2 g, 6.7 mmol, 73%) was dissolved in ice-cold DMF (50 mL) and neutralized with triethylamine (1.9 mL, 13.5 mmol).

Z-Gly-OSu (5) (2.05 g, 6.7 mmol) was added to the tripeptide solution and the reaction mixture was stirred overnight at room temperature. Evaporation of the solvent gave a residue which was dissolved in butan-1-ol saturated with 5% aqueous acetic acid (100 mL), washed with 5% aqueous acetic acid (3 x 100 mL) and water (1 x 100 mL) and taken to dryness. The product was purified by flash chromatography on the Chromatospac-Prep 100 Apparatus [80 g of Silica Gel F 60, methylene chloride-methanol (92.5:7.5 v/v), flow rate 50 mL/min, fraction volume 50 mL]. Fractions containing single spot material by t.l.c. (solvent systems I, II, and IV) were pooled, evaporated to dryness and the residue was precipitated from methanol with ether (2.8 g, 62% as mono-hydrate); m.p. 95–100° (gradual softening); $[\alpha]_D^{25} -40.6°$ (*c* 1.0, methanol).

Anal. calc. for $C_{27}H_{38}N_8O_{11} \cdot H_2O$ (668.63): C 48.50, H 6.03, N 16.76.
Found: C 48.37, H 5.87, N 16.44.

Amino acid ratios in acid hydrolysate: Pro 1.10, Glu 0.98, Gly 1.00 (Arg + Orn) 0.95.

*Z-Gly-Glu(*NHNHBoc*)-Pro-Arg(NO$_2$)-OMe (VII).* A mixed anhydride was prepared from Z-Gly-Glu-Pro-Arg(NO$_2$)-OMe (2.2 g, 3.38 mmol) in

H_4-furan (30 mL) with *N*-methylmorpholine (0.38 mL, 3.38 mmol) and isobutylchloroformate (0.44 mL, 3.38 mmol) at −15° for 2 min and Boc-$NHNH_2$ (0.67 g, 5.07 mmol) added.

The reaction mixture was stirred for 30 min at −15°, for 12 h at room temperature and evaporated to dryness. The residue was dissolved in butan-1-ol, washed, dried, evaporated to dryness and precipitated from methanol with ether. The product (2.2 g, 85%) contained some minor contaminants (t.l.c. in solvent system IV) but was used without further purification. A sample (0.2 g) for the analysis was purified by flash chromatography on the Chromatospac-Prep 100 Apparatus [90 g of Silica Gel F 60, methylene chloride-methanol (98.5:1.5 v/v), flow rate 50 mL/min, fraction volume 50 mL]. Yield 0.14 g (70%), m.p. 91–93° (softening); $[\alpha]_D^{25°}$ −33.3° (*c* 1.0, methanol); single spot by t.l.c. in solvent systems I, II, and IV.

Anal. calc. for $C_{32}H_{48}N_{10}O_{12}$ (764.77): C 50.25, H 6.33, N 18.31.

Found: C 49.80, H 6.44, N 17.76.

Amino acid ratios in acid hydrolysate: Pro 1.00, Glu 1.05, Gly 1.00 (Arg + Orn) 0.93.

Z-Gly-Glu(NHGlc)-Pro-Arg(NO_2)-OMe (VIII). Z-Gly-Glu(NHNH-Boc)-Pro-Arg(NO_2)-OMe (2.0 g, 2.62 mmol) was dissolved in 95% aqueous trifluoroacetic acid (8 mL). After 1 h excess ether was added and the resulting precipitate was collected, thoroughly washed with ether and dried over potassium hydroxide pellets.

The isolated material (1.8 g, 88%, single spot by t.l.c. in solvent system I) was dissolved in DMF (20 mL), cooled to −15°, and 3.88 M HCl in dioxane (2.38 mL, 9.22 mmol) and *tert.*-butyl nitrite (0.3 mL, 2.54 mmol) were added. After stirring at −15° for 15 min the solution was cooled to −50° and triethylamine (1.61 mL, 11.53 mmol) was added followed by a precooled solution of 2-amino-2-deoxy-β-D-glucopyranose hydrochloride (1.0 g, 4.64 mmol) in 50% aqueous DMF (20 mL) containing triethylamine (0.65 mL, 4.64 mmol).

The reaction mixture was stirred 3 days at 5°, an additional 24 h at room temperature and evaporated to dryness. The residue was dissolved in butan-1-ol, previously saturated with 5% aqueous acetic acid, and washed several times with 5% aqueous acetic acid, saturated with butan-1-ol, and finally with water saturated with butan-1-ol. The organic layer was evaporated to dryness and the residue precipitated from methanol with ether. The crude product (1.6 g) was purified by flash chromatography on a LiChroprep RP-8 column (2.5 x 15 cm, flow rate 30 mL/min, fraction volume 30 mL) eluted with 2000 mL acetonitrile-water (1:9 v/v) followed by 1000 mL acetonitrile-water (2:8 v/v). Fractions revealing single spot material on RP-8 t.l.c. (solvent system: acetonitrile-water, 4:6 v/v) were pooled and evaporated to dryness (1.17 g, 60% as di-hydrate), m.p. 102–104° (softening), 176–177° dec.; $[\alpha]_D^{25°}$ −19.3° (*c* 1.0, methanol).

Anal. calc. for $C_{33}H_{49}N_9O_{15} \cdot 2H_2O$ (847.79): C 46.75, H 6.30, N 14.87.

Found: C 47.03, H 6.08, N 14.93.

Amino acid ratios in acid hydrolysate: Pro 1.07, Glu 0.99, Gly 1.01 (Arg + Orn) 0.94 (92% as di-hydrate).

H-Gly-Glu(NHGlc)-Pro-Arg-OH (IX). A sample of Z-Gly-Glu(NHGlc)-Pro-Arg(NO_2)-OMe (0.62 g, 0.73 mmol) was dissolved in a mixture (20 mL) of dioxane-water (4:1 v/v) and 1 N NaOH (0.75 mL) was added dropwise. After 1 h stirring the solution was cooled to 0°, neutralized with 1 N HCl and evaporated to dryness. The residue was dissolved in a mixture (60 mL) of methanol-20% acetic aqueous acid (1:1 v/v) and hydrogenated for 5 h. The catalyst was removed by filtration and the solution was concentrated *in vacuo* to remove methanol and finally lyophilized.

The crude product was chromatographed on a CM 52 column and desalted (twice) on a Sephadex G 10 column as previously described in the preparation of H-Gly-Gln-Pro-Arg-OH (IV). Yield 0.29 g (50% as di-acetate, tri-hydrate); $[\alpha]_D^{25°}$ −40.8° (*c* 1.0, 5% aqueous acetic acid). The purity of IX was checked by analytical RP-HPLC (conditions as in Fig. 6).

Anal. calc. for $C_{24}H_{42}N_8O_{11} \cdot 2CH_3COOH \cdot 3H_2O$ (792.7): C 42.42, H 7.12, N 14.14.

Found: C 42.73, H 7.12, N 14.16.

Amino acid ratios in acid hydrolysate:

Pro 1.00, Glu 1.00, Gly 0.96, Arg 1.00 (99% as di-acetate, tri-hydrate).

Boc-Gln-Pro-OBzl (X). Triethylamine (9.8 mL, 70 mmol) and Boc-Gln-OSu (7) (9.96 g, 29 mmol) were added to an ice cold suspension of H-Pro-OBzl hydrochloride (6) (8.46 g, 35 mmol) in H_4-furan (250 mL) and the reaction mixture was stirred at room temperature for 24 h. The solvent was evaporated and the residue was taken up in ethyl acetate, washed (0.1 M $KHSO_4$, 5% $NaHCO_3$, and water), dried, and evaporated to dryness. The residue was dissolved in ethyl acetate, filtered to remove some insoluble material, and precipitated with light petroleum. The resulting oil crystallized on standing in the refrigerator (4.15 g, 33%); m.p. 129–131°; $[\alpha]_D^{25°}$ −82.8° (*c* 1.0, methanol); single spot by t.l.c. in solvent systems I, II, III and IV.

Anal. calc. for $C_{22}H_{31}N_3O_6$ (433.48): C 60.95, H 7.21, N 9.69.

Found: C 60.70, H 7.20, N 9.64.

Amino acid ratios in acid hydrolysate: Pro 0.98, Glu 1.02.

Z-Gly-Gln-Pro-OBzl (XI). Compound X (2.8 g, 6.5 mmol) was deblocked with 1.5 N HCl in ethyl acetate (120 mL) for 45 min. Addition of excess ether gave a precipitate which was collected and dried (1.92 g, 5.2 mmol, 80%). The product was dissolved in ice-cold DMF (100 mL), and triethylamine (0.73 mL, 5.2 mmol) and Z-Gly-OSu (5) (1.59 g, 5.2 mmol) were added.

After 48 h the reaction mixture was evaporated to dryness and the residue dissolved in ethyl acetate, washed, dried, and precipitated with light petroleum. The resulting oil was purified by flash chromatography on the Chromatospac-Prep 100 Apparatus [80 g of Silica Gel F 60, methylene chloride-methanol (98:2 v/v) flow rate 50 mL/min, fraction volume 50 mL]. Fractions containing single spot material by t.l.c. (solvent system IV) were combined and evaporated to dryness (1.84 g, 65%); m.p. 65–67° (softening); $[\alpha]_D^{25°}$ −66.9° (*c* 1.07, methanol).

Anal. calc. for $C_{27}H_{32}N_4O_7 \cdot H_2O$ (542.55): C 59.77, H 6.32, N 10.33.

Found: C 60.52, H 6.10, N 10.45.

Amino acid ratios in acid hydrolysate: Pro 0.98, Glu 1.02, Gly 1.00.

Z-Gly-Gln-Pro-Arg(NO_2)-NHGlc (XII). Hydrazine hydrate (0.75 mL) was added to a solution of Z-Gly-Gln-Pro-OBzl (0.51 g, 0.95 mmol) in methanol (15 mL). After 6 days excess ether was added and the resulting precipitate was collected, reprecipitated from methanol with ether (0.4 g, 94%, single spot by t.l.c. in solvent system I) and used without further purification to acylate H-Arg(NO_2)-NHGlc.

The tripeptide hydrazide (0.4 g, 0.89 mmol) was dissolved in DMF (3 mL), cooled to −20°, and 3.88 N HCl in dioxane (0.92 mL, 3.56 mmol) and *tert.*-butyl nitrite (0.126 mL, 1.06 mmol) were added. After 20 min stirring at −20° the mixture was cooled to −50° and triethylamine (0.5 mL, 3.56 mmol) was added.

Boc-Arg(NO_2)-NHGlc hemi-hydrate (0.52 g, 1.06 mmol) was selectively deblocked by trifluoroacetic acid treatment as already described (1), dissolved in DMF (5 mL) containing triethylamine (0.148 mL, 1.06 mmol), cooled to −20° and added to the tripeptide azide solution described above. The reaction mixture was stirred for 5 days at 4°, filtered to remove some insoluble material, and ether was added to the solution. The resulting precipitate was collected by centrifugation, dissolved in methanol and reprecipitated with ether. The crude material (0.55 g) was purified by flash chromatography on a LiChroprep RP-8 column (2.5 × 14 cm, acetonitrile-water, 2:8 v/v, flow rate 30 mL/min, fraction volume 30 mL). Fractions containing single spot material by t.l.c. (solvent system I) and RP-8 t.l.c. (acetonitrile-water, 30:70 v/v as the eluant) were combined and evaporated to dryness. The residue was precipitated from methanol with ether (0.45 g, 61% as di-hydrate); m.p. 145–148° (softening), 200–202° dec.; $[\alpha]_D^{25°}$ −28.5° (*c* 1.07, methanol).

Anal. calc. for $C_{32}H_{48}N_{10}O_{14} \cdot 2H_2O$ (832.84): C 46.15, H 6.29, N 16.82.

Found: C 45.91, H 6.14, N 16.41.

Amino acid ratios in acid hydrolysate: Pro 1.05, Glu 0.97, Gly 0.98 (Arg + Orn) 0.95.

H-Gly-Gln-Pro-Arg-NHGlc (XIII). A solution of Z-Gly-Gln-Pro-Arg(NO_2)-NHGlc · $2H_2O$ (0.33 g,

0.4 mmol) in 50 mL methanol-20% aqueous acetic acid (1:1 v/v) was hydrogenated for 3 h, the catalyst was removed by filtration and the solution was concentrated to remove the organic solvent and finally lyophilized. The residue was chromatographed on a CM 52 column and desalted on a Sephadex G 10 column as previously described in the preparation of compound IV. Yield 0.165 g (52% as di-acetate, tri-hydrate); $[\alpha]_D^{25}$ −63.7° (*c* 1.0, 5% aqueous acetic acid). The purity of XIII was checked by analytical RP-HPLC (conditions as in Fig. 6).

Anal. calc. for $C_{24}H_{43}N_9O_{10} \cdot 2CH_3COOH \cdot 3 H_2O$ (791.81): C 42.47, H 7.25, N 15.92.

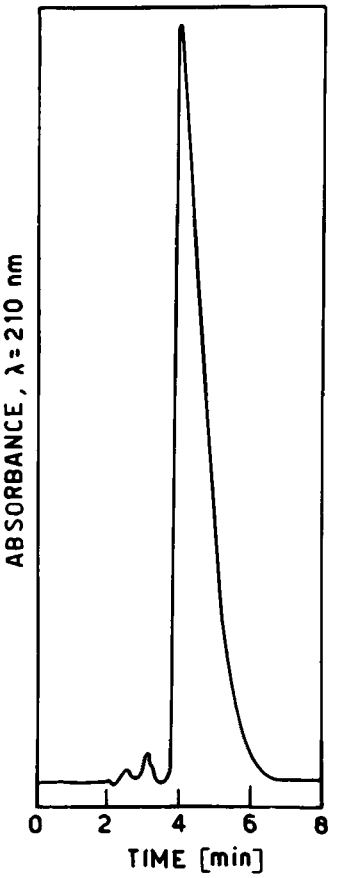

FIGURE 6

Analytical HPLC of rigin. Column: Merck Hibar LiChrosorb RP-8, 7 μm (4 × 250 mm); Load 100 μg; Eluant: (A) 20% propan-2-ol in B, (B) 0.1% trifluoroacetic acid; Isocratic elution 75% A in B; Flow rate 1.2 mL/min; Sensitivity 1.28 AUFS.

Found: C 42.57, H 7.38, N 16.09.

Amino acid ratios in acid hydrolysate: Pro 0.99, Glu 0.99, Gly 1.00, Arg 1.02 (99% as di-acetate, tri-hydrate).

Z-Gln-Pro-N_2H_2-Boc(XIV). The mixed anhydride prepared from Z-Gln-OH (11) (14.85 g, 53 mmol) in H_4-furan (150 mL) with *N*-methylmorpholine (5.9 mL, 53 mmol) and isobutylchloroformate (6.87 mL, 53 mmol) at −15° for 2 min was added to a cooled (−15°) solution of H-Pro-N_2H_2-Boc hydrochloride (14) (14 g, 53 mmol) and triethylamine (7.42 mL, 53 mmol) in H_4-furan (120 mL). After 1 h at −15° and 12 h at room temperature the solvent was evaporated, and the residue was taken up in ethyl acetate, washed, and dried. Evaporation to a small volume followed by addition of ether gave a precipitate, which was collected and dried (11.5 g, 44%); m.p. 82–85° (gradual softening), 145° (melt); $[\alpha]_D^{25}$ −106° (*c* 1.0, methanol); single spot by t.l.c. in solvent sytems I, II, III, and IV.

Anal. calc. for $C_{23}H_{33}N_5O_7 \cdot H_2O$ (509.53): C 54.21, H 6.92, N 13.75.

Found: C 54.71, H 6.65, N 13.84.

Z-Gln-Pro-Arg(NO_2)-OBzl (XV). Z-Gln-Pro-N_2H_2-Boc·H_2O (1.02 g, 2 mmol) was deprotected with 90% aqueous trifluoroacetic acid (4 mL), converted into the corresponding azide in the usual way [DMF (30 mL), 3.88 N HCl in dioxane (2.06 mL, 8 mmol), *tert.*-butyl nitrite (0.26 mL, 2.2 mmol), triethylamine (1.12 mL, 8 mmol)] and reacted with H-Arg(NO_2)-OBzl hydrochloride (8) (0.76 g, 2 mmol) in DMF (30 mL) containing triethylamine (0.28 mL, 2 mmol).

After 3 days at 5° and 24 h at room temperature the reaction mixture was taken to dryness and the residue was taken up in ethyl acetate, washed and dried. After evaporation of the solvent the product was precipitated from methylene chloride with ether, re-dissolved in methanol, filtered to remove some insoluble material and re-precipitated with ether (1.3 g, some minor contaminants by t.l.c. in solvent systems I, II, and III). The crude product was purified by flash chromatography on the Chromatospac-Prep 100 Apparatus [70 g of Silica Gel F 60, methylene chloride-methanol, 95:5 v/v, flow

rate 50 mL/min, fraction volume 50 mL]. Fractions containing single spot material by t.l.c. (solvent systems I, II, and III) were combined and evaporated to dryness (1.15 g, 84% as mono-hydrate); m.p. 75–78° (gradual softening), 165–168° dec.; $[\alpha]_D^{25°}$ −57.6° (*c* 1.0, methanol).

Anal. calc. for $C_{31}H_{40}N_8O_9 \cdot H_2O$ (686.68): C 54.22, H 6.17, N 16.32.

Found: C 54.59, H 6.09, N 16.26.

Amino acid ratios in acid hydrolysate: Pro 0.98, Glu 1.00 (Arg +) 0.96.

Alternatively the title compound was prepared by reacting the mixed anhydride prepared from Z-Gln-OH (11) (0.56 g, 2.0 mmol) as described in the preparation of XIV with H-Pro-Arg(NO_2)-OBzl di-trifluoroacetate (9, 10) (1.23 g, 2.0 mmol) in H_4-furan (25 mL) containing triethylamine (0.56 mL, 4.0 mmol). After stirring 1 h at −15° and overnight at room temperature the solvent was removed and the residue was worked up as previously described (0.82 g, 60% as mono-hydrate); m.p. 75–78° (gradual softening), 163–165° dec.; $[\alpha]_D^{20°}$ − 57.4° (*c* 1.0, methanol), analytically and chromatographically indistinguishable from the previous material.

D-Gluconyl-Gly-Gln-Pro-Arg(NO_2)-OBzl (XVI). D-Gluconyl-Gly-NHNH-Boc (12) (0.382 g, 1.04 mmol) was dissolved in 2 N HCl (1.5 mL). After 2 h at room temperature the solution was lyophilized and the product (0.31 g, 100%, single spot by t.l.c. in solvent system I) was dissolved in DMF (3 mL). The solution was cooled to −20° and 3.88 N HCl in dioxane (1.07 mL, 4.16 mmol) and *tert.*-butyl nitrite (0.15 mL, 1.25 mmol) were added. After 20 min stirring at −20° the mixture was cooled to −50° and added dropwise to a stirred solution of H-Gln-Pro-Arg(NO_2)-OBzl obtained by treatment (15 min at 20°) of the corresponding N^{α}-benzyloxycarbonyl derivative (XV) (0.71 g, 1.04 mmol) with 33% HBr in acetic acid (10 mL), evaporation to dryness and solution of the residue in ice-cold DMF (12 mL) containing triethylamine (0.29 mL, 2.08 mmol).

After 4 days the reaction mixture was evaporated to dryness and the residue was applied to a LiChroprep RP-8 column (3.3 × 12.5 cm) and purified by flash chromatography (acetonitrile-water, 30:70 v/v,flow rate 30 mL/min, fraction volume 30 mL). Fractions containing single spot material by t.l.c. (solvent systems I and II) and RP-8 t.l.c. (acetonitrile-water, 40:50 v/v as the eluant) were pooled and evaporated to dryness (0.18 g, 22%), m.p. 120–122° (softening); $[\alpha]_D^{25°}$ −37.7° (*c* 1.01, methanol).

Anal. calc. for $C_{31}H_{47}N_9O_{14} \cdot 2H_2O$ (805.80): C 46.20, H 6.38, N 15.64. Found: C 46.45, H 6.25, N 15.38.

Amino acid ratios in acid hydrolysate: Pro 1.00, Glu 1.00, Gly 1.00 (Arg + Orn) 0.98.

D-Gluconyl-Gly-Gln-Pro-Arg-OH (XVII). Compound XVI (0.169 g, 0.21 mmol) was dissolved in a mixture (50 mL) of methanol-20% aqueous acetic acid (1:1 v/v) and hydrogenated for 4 h. Purification of the deblocked peptide on CM 52 column and desalting on Sephadex G 10 were carried out as previously described in the preparation of H-Gly-Gln-Pro-Arg-OH (IV). Yield 0.137 g (90% as mono-acetate, di-hydrate); $[\alpha]_D^{25°}$ −43.3° (*c* 1.0, 5% aqueous acetic acid).

Anal. calc. for $C_{24}H_{42}N_8O_{12} \cdot CH_3COOH \cdot 2H_2O$ (730.65): C 42.74, H 6.90, N 15.34.

Found: C 42.40, H 6.91, N 15.82.

Amino acid ratios in acid hydrolysate: Pro 0.99, Glu 1.00, Gly 1.01, Arg 1.00 (98% as mono-acetate, di-hydrate). Analytical RP-HPLC of the isolated product is shown in Fig. 7.

Z-Glu(OBu^t)-Pro-Arg(NO_2)-OBzl (XVIII). The mixed anhydride was prepared from Z-Glu (OBu^t)-OH (4) (2.83 g, 8.4 mmol) in H_4-furan (30 mL) with *N*-methylmorpholine (0.93 mL, 8.4 mmol) and isobutylchloroformate (1.09 mL, 8.4 mmol) at −15° for 5 min and added to an ice-cold solution of H-Pro-Arg(NO_2)-OBzl di-trifluoroacetate (9, 10) (5.0 g, 7.9 mmol) and triethylamine (2.21 mL, 15.8 mmol) in H_4-furan (80 mL).

After 10 min at −15° and 2 h at room temperature the solvent was removed and the residue was taken up in ethyl acetate, washed, dried, and concentrate to small volume. Addition of light petroleum gave an oil which crystallized by trituration with light petroleum (5 g, 83%). The crude product was purified by flash

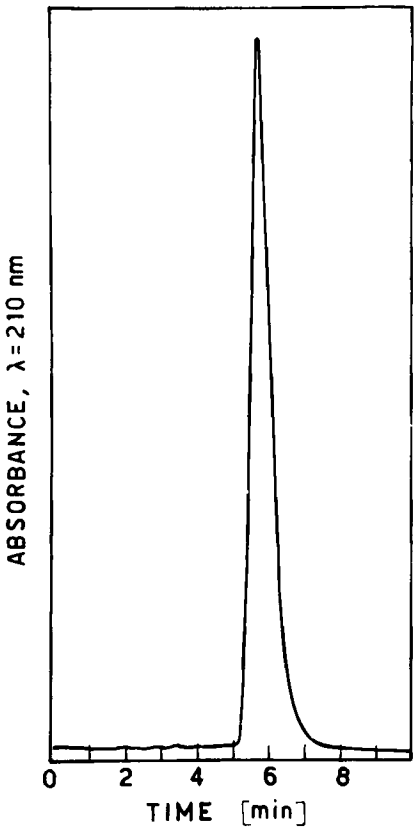

FIGURE 7

Analytical HPLC of D-gluconyl-rigin. Column: as in Fig. 6; Load: 100 μg; Eluant: (A) 20% methanol in B, (B) 0.1 M $NH_4H_2PO_4$, pH 4.6; Isocratic elution 10% A in B; Flow rate 1 mL/min; Sensitivity 1.28 AUFS.

chromatography on the Chromatospac-Prep 100 Apparatus [80 g of Silica Gel F 60, methylene chloride – methanol (99:1 v/v), flow rate 50 mL/min, fraction volume 50 mL]. The desired fractions were combined and evaporated to dryness (4.3 g, 75%, some minor contaminants by t.l.c. in solvent system I). An aliquot (1 g) of the crude material was further purified by flash chromatography on the Chromatospac-Prep 100 Apparatus [80 g Silica Gel F 60, ethyl acetate – acetone (95:5 v/v), flow rate 50 mL/min, fraction volume 50 mL].

Fractions containing single spot material (t.l.c. in solvent systems I, II, III and VI) were pooled and evaporated to dryness (0.26 g, 26%); m.p. 90° (softening); $[\alpha]_D^{25}$ −48.9° (*c* 1.0, methanol).

Anal calc. for $C_{35}H_{47}N_7O_{10}$ (725.77): C 57.92, H 6.53, N 13.51.

Found: C 57.43, H 6.70, N 13.29.

H-Gly-Glu-Pro-Arg-OH (XXII). Crude Z-Glu (OBu^t)-Pro-Arg(NO_2)-OBzl (3.3 g, 4.6 mmol) was dissolved in 33% HBr in acetic acid (10 mL) and stirred for 20 min. Addition of excess ether gave a precipitate which was collected, washed several times with ether, dried over KOH pellets, and dissolved in DMF (50 mL) containing triethylamine (1.29 mL, 9.2 mmol). Z-Gly-OSu (5) (1.4 g, 4.6 mmol) was added and the reaction mixture was stirred overnight at room temperature and concentrated to small volume. Excess ether was then added and the resulting precipitate was dissolved in methylene chloride, washed, dried, and reprecipitated with ether (2.52 g, 75%). Attempts to purify the crude product [m.p. 105° (softening), $[\alpha]_D^{25}$ −48° (*c* 0.99, methanol), some contaminants by t.l.c. in solvent systems I and II] failed and the product was directly submitted to the deblocking step.

An aliquot of Z-Gly-Glu-Pro-Arg(NO_2)-OBzl (0.5 g, 0.64 mmol) was dissolved in methanol (30 mL), diluted with 20% aqueous acetic acid (30 mL) and hydrogenated for 2½ h. The catalyst was removed by filtration, the solution was taken to dryness and the residue was chromatographed on a CM 52 column and desalted on a Sephadex G 10 column as previously described in the preparation of compound IV. Yield 0.26 g (82% as di-hydrate); $[\alpha]_D^{25}$ − 81.2° (*c* 1.0, 5% aqueous acetic acid). The purity of XXII was checked by RP-HPLC (conditions as in Fig. 7).

Anal. calc. for $C_{18}H_{31}N_7O_7 \cdot 2H_2O$ (493.49): C 43.81, H 7.15, N 19.87.

Found: C 44.14, H 7.01, N 19.65.

Amino acid ratios in acid hydrolysate: Gly 1.01, Glu 0.98, Pro 1.00, Arg 1.00 (98% as di-hydrate).

Boc-Glu(OBzl)-Pro-Arg(NO_2)-OBzl (XIX). The mixed anhydride was prepared from Boc-Glu (OBzl)-OH (2.3 g, 4.4 mmol), obtained by partitioning the corresponding dicyclohexyl-

ammonium salt (15), between citric acid and ethyl acetate, with *N*-methylmorpholine (0.49 mL, 4.4 mmol) and isobutylchloroformate (0.55 mL, 4.2 mmol) in H_4-furan (30 mL), at $-15°$ for 5 min and added to an ice-cold solution of H-Pro-Arg(NO_2)-OBzl di-trifluoroacetate (9, 10) (2.66 g, 4.2 mmol) and triethylamine (1.18 mL, 8.4 mmol) in H_4-furan (50 mL).

After 30 min at 0° and 60 min at room temperature the solvent was removed and the residue was taken up in ethyl acetate, washed, dried, and concentrated to small volume. Addition of light petroleum gave an oil which crystallized by trituration with light petroleum (1.83 g, 60%); m.p. 108–110°; $[\alpha]_D^{20}$ −50.2° (*c* 1.06, methanol); single spot by t.l.c. in solvents I, II and III.

Anal. calc. for $C_{35}H_{47}N_7O_{10}$ (725.77): C 57.92, H 6.53, N 13.51.

Found: C 58.19, H 6.70, N 13.77.

Amino acid ratios in acid hydrolysate: Pro 1.10, Glu 1.00 (Arg + Orn) 1.01.

D-Gluconyl-Gly-Glu(OBzl)-Pro-Arg(NO_2)-OBzl (XXI). Boc-Glu(OBzl)-Pro-Arg(NO_2)-OBzl (0.77 g, 1.06 mmol) was dissolved in 1.5 N HCl in ethyl acetate (20 mL). After 60 min the resulting precipitate was collected, washed with ethyl acetate and ether, dried *in vacuo* and dissolved in DMF (30 mL) containing triethylamine (0.3 mL, 2.12 mmol) and cooled to −15°.

Simultaneously *tert.*-butyl nitrite (0.14 mL, 1.17 mmol) was added to a solution, cooled to −15°, of D-gluconyl-Gly-N_2H_3 hydrochloride (12) (0.32 g, 1.06 mmol) in DMF (30 mL) containing 3.53 M HCl in dioxane (1.5 mL, 4.24 mmol).

The mixture was stirred at −15° for 15 min, then cooled to −50° and triethylamine (0.74 mL, 5.3 mmol) was added. The precooled solution of the N^{α}-deprotected tripeptide benzylester was added to the azide solution and the reaction mixture stirred 4 days at 5°, an additional 24 h at room temperature and evaporated to dryness. The residue was precipitated from methanol with a mixture of ether and ethyl acetate (1:1 v/v). The crude material (0.77 g, 85%) was purified by flash chromatography [LiChroprep RP-8 column (3.3 x 12.5 cm), acetonitrile-water (30:80 v/v), flow rate 50 mL/min, fraction volume 50 mL].

Fractions containing single spot material by t.l.c. (solvent system I) were pooled and evaporated to dryness. The residue was precipitated from methylene chloride with light petroleum (0.42 g, 45%); m.p. 80–82° (softening), 200–205° dec.; $[\alpha]_D^{20}$ −32.2° (*c* 0.9, methanol).

Anal. calc. for $C_{38}H_{52}N_8O_{15} \cdot 1/2\,H_2O$ (869.84): C 52.47, H 6.14, N 12.88.

Found: C 52.45, H 6.19, N 12.85.

Amino acid ratios in acid hydrolysate: Pro 0.98, Glu 1.01, Gly 1.00 (Arg + Orn) 0.97.

D - Gluconyl - Gly - Glu - Pro - Arg - OH (XXIII). D-Gluconyl-Gly-Glu(OBzl)-Pro-Arg(NO_2)-OBzl hemi-hydrate (0.25 g, 0.287 mmol) was hydrogenated for 6 h in 50% methanolic acetic acid (4 mL). The supernatant was decanted, the catalyst thoroughly washed (3 x 4 mL) with 50% aqueous methanol, the solution and washings combined, filtered (Millipore LSWP, 5.0 μm) and evaporated to dryness. The crude material was chromatographed on a CM 52 column and desalted on a Sephadex G 10 column as previously described in the preparation of compound IV. Fractions containing single spot material (solvent systems I and II) were combined and lyophilized (93 mg, 45% as mono-hydrate); $[\alpha]_D^{25}$ −48.6° (*c* 1.0, 5% aqeuous acetic acid).

Anal. calc. for $C_{24}H_{41}N_7O_{13} \cdot CH_3COOH \cdot H_2O$ (713.64): C 43.75, H 6.64, N 13.74.

Found: C 43.79, H 6.72, N 13.84.

Amino acid ratios in acid hydrolysate: Gly 0.98, Glu 1.01, Pro 0.98, Arg 1.02 (98% as mono-acetate, mono-hydrate).

RESULTS AND DISCUSSION

Tuftsin was found to augment the phagocytic capacity of mouse peritoneal macrophages by 34%. Of all the peptides tested, rigin is the only compound that enhanced the activity of the cell to the same extent as tuftsin, when maximal effect was observed at identical peptide concentrations (5×10^{-8} M) (Fig. 8). Rigin, however, is capable of efficiently displacing tritiated tuftsin from macrophages only when

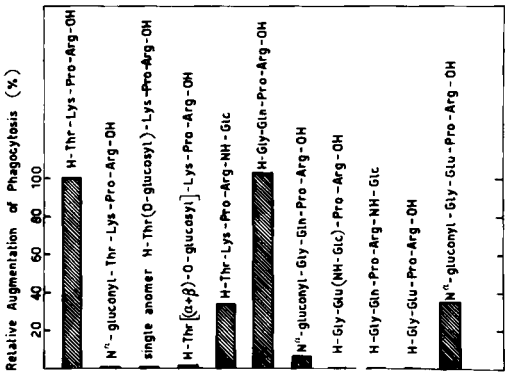

FIGURE 8

Effect of peptides on augmentation of phagocytosis. Peptides were applied to macrophages at concentration of 5×10^{-8} M. Uptake of ^{51}Cr-IgG-SRBC by the cells was measured. 100% augmentation refers to the augmentation of phagocytosis of a tuftsin concentration of 5×10^{-8} M. Zero stimulation refers to phagocytosis by macrophages without tuftsin.

applied at high concentration ($\sim 10^{-4}$ M) (Fig. 9).

The peptide H-Thr[(α + β)-*O*-glucosyl]-Lys-Pro-Arg-OH (1) was found to displace ^{3}H-tuftsin even better than tuftsin (Fig. 9).

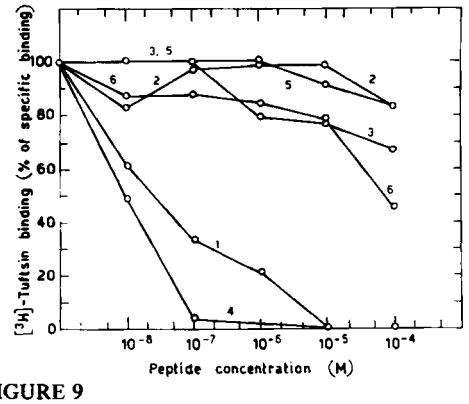

FIGURE 9

Displacement of [^{3}H]-tuftsin binding to macrophages by tuftsin, rigin and by different tuftsin analogs. Macrophages were incubated with tritiated tuftsin. The different peptides were then added at the indicated concentrations. 100% specific binding refers to the amount of [^{3}H]-tuftsin displaced by 10 μM tuftsin. 1) H-Thr-Lys-Pro-Arg-OH; 2) N^{α}-Gluconyl-Thr-Lys-Pro-Arg-OH; 3) H-Thr[O-glucosyl)-Lys-Pro-Arg-OH (single anomer); 4) H-Thr-[(α + β)-*O*-glucosyl]-Lys-Pro-Arg-OH; 5) H- Thr-Lys-Pro-Arg-NH-Glc; 6) H-Gly-Gln-Pro-Arg-OH.

However, this peptide lacks the ability to stimulate phagocytosis (Fig. 8). To test whether this sugar-containing analog is an inhibitor of tuftsin, it was applied to phagocyting macrophages at concentration of 5×10^{-8} M together with tuftsin or rigin (at concentrations ranging from 10^{-8} M to 10^{-5} M). No inhibition of the stimulatory effects of either tuftsin or rigin was detected. The peptides H-Thr-Lys-Pro-Arg-NH-Glc (1) and N^{α}-gluconyl-Gly-Glu-Pro-Arg-OH are capable of slightly enhancing (~ 30%, as compared to tuftsin or rigin) phagocytosis (Fig. 8). They could not, however, displace ^{3}H-tuftsin from macrophages (the former peptide (Fig. 9), the latter not shown).

The results of both binding and phagocytosis assays point, again, to the specificity of tuftsin-macrophage association and its consequent cell activation (16). Thus, glucosylation of the tuftsin molecule, at various positions, led to inactive analogs. Similarly, attachment of sugar moieties to the rigin molecule led to inactive derivatives. It seems to us that rigin may activate macrophages through binding to receptors which are not related to the tuftsin receptor. Direct binding of labeled rigin and competition between this derivative and tuftsin may clear this point. It is worth noting here that rigin contains a Pro-Arg sequence which has been proposed to be a signal-unit in various biologically active peptides (17).

The (α + β)-*O*-glucosyl-tuftsin was shown to displace ^{3}H-tuftsin efficiently from macrophages, though it is devoid to phagocytosis-related activity. Several analogs of tuftsin were found to be devoid of similar *in vitro* activity as well. They proved, however, to be active and to possess various tuftsin-like features *in vivo* (16).

The tuftsin-moiety is located within the CH2 domain of the Fc-fragment of γ-globulin molecule, in the vicinity of the carbohydrate binding site (18):

```
. . . His-Asn-Ala-Lys-Thr-Lys-Pro-Arg-Glu-
                289       292
                          carbohydrate
                              |
                Gln-Gln-Tyr-Asx . . .
```

We have previously synthesized the dodecapeptide and octapeptide which correspond,

respectively, to amino acid residues 285–296 and 289–296 of IgG. These peptides were shown to attach to mouse peritoneal macrophages specifically and to enhance their phagocytic capacity to the same extent as tuftsin (19). We intend to extend, using the synthetic methodology described in this paper, these sequences so as to include as Asx-297-sugar moiety and to study their binding to macrophages and their capacity to activate the cells. This may shed a certain light on the possible precursor molecule of tuftsin and the Fc-like nature of its receptor site (19).

Note added in proof. While this paper was in press ^{1}H-n.m.r. studies on the single diastereoisomer and on the ($\alpha + \beta$) mixture of *O*-glucosyl-tuftsin allowed us to assign the β-configuration to the *O*-glycosidic linkage in the single diastereoisomer, and to estimate 4:1 α:β ratio in the mixture. Details of the n.m.r. experiments will be reported elsewhere.

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