change chromatography on SE- and DEAE-Sephadex). Titration curves of 10 mg protein in 20 mmol/l sodium hydroxide were obtained between pH 11.6 and 3.5 by down-scale titration with 25 mmol/l sulfuric acid (Radiometer titration assembly). Native and substituted proteins were hydrolyzed by trypsin and pronase (Merck), according to NAKAYA et al.[11], followed by TLC (silica gel G Merck, butanol (−1) 4 – acetic acid 1 – water 1 and phenol 3 – water 1) and disc electrophoresis (7.5% polyacrylamide gel, 7 V/cm, vertical rods).

Results. After a reaction period of 30 min, red products appear. They could not be isolated because of decomposition on evaporation. Their almost uniform spectra resemble those of complexes formed by buffered solutions of amino acids between pH 5.5 and 7.5 with an excess of pBQ in organic solvents[9]. The reaction is first order with respect to both components, suggesting a 1:1 interaction. The spectral bands also agree with the maxima of chloranil amino acid charge transfer complexes[12] and probably en route formed monosubstituted pBQ compounds[8], but they entirely differ from the various colours of isolated 2-(amino acid)-quinones[5,6]. The number of the groups reacting with pBQ within 30 min corresponds to the calculated value only in small stretched molecules (table). In the native state, 4 lysine residues in ribonuclease, 1 in insulin and 16 in albumin are not accessible, human fibrinogen, casein, and gelatin even showing less reactivity.

Reaction of proteins at 25 °C within 30 min, see 'methods'

Original protein or amino acid	max (nm)	Number of reactive sites α-NH_2	ε-NH_2	Indole	ε_{492} calc.	(cm^2/μmol) found
α-Amino acids[a]	490	1	–	–	–	2.75
Nα-Acetyllysine	490	–	1	–	–	1.65
Lysine	490	1	1	–	4.40	4.40
Tryptophan	500	1	–	1	–	3.55
Pepsin C	492	1	4	6	14.15	14.10
Insulin						
oxidized a-chain	490	1	–	–	2.75	2.75
denatured	485	2	1	–	7.15	7.15
native	485	2	1	–	7.15	5.50
Ribonuclease						
oxidized	490	1	10	–	19.25	19.30
native	485	1	10	–	19.25	12.60
Albumin	490	1	56	1	95.95	69.50

[a] Except tryptophan, lysine, proline and cysteine.

Treatment lasting for 2 h leads to dark solutions with absorption maxima between 320 and 350 nm, known from 2,5-disubstituted-pBQ[8], followed by peaks at 230–250 nm after a period of 24 h, indicating the formation of 2-monosubstituted-pBQ[5]. The isolated pBQ protein complexes form hygroscopic brown to red solids, which are throughout more soluble at pH 7.5 and less precipitable by trichloracetic acid than their native analogues. This may be attributed to a higher negative charge by the introduction of quinone residues as indicated by titration curves displaying more titratable groups between pH 8.0 and 10.0, but less above 10.6. This suggests a substitution of side chain amino groups. As compared to the native proteins, the complexes show a 1.3–1.4 times higher anodic mobility in electrophoresis at pH 8.6 and equal velocity at pH 6.5. They are less retarded on SE-Sephadex at pH 7.0, but more on DEAE-Sephadex at pH 8.0, where pBQ-casein and -albumin cannot be eluted by 50 mmol/l triethanolamine. At 25 °C the complexes are stable for at least 10 days against 100 mmol/l sodium hydroxide and sulfuric acid, 5 mol/l urea and up to 50 g/l Tween 20. Pronase exerts identical effects on native and pBQ marked proteins, while peptide maps and disc electrophoretic patterns after digestion with trypsin show reduced cleavage by preceeding treatment with pBQ. Thus blocking of lysine prevents splitting of the b-chain of insulin and the formation of tryptic cores in the hydrolysis of substituted albumin, casein and insulin. As revealed by gel filtration and SDS disc electrophoresis, native proteins and pBQ protein complexes possess the same molecular weights roughly excluding pBQ cross-linking between molecules.

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Synthesis of Bradykinin Potentiating Pentapeptide (BPP_{5a})

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Summary. This synthesis is especially suitable for production of highly-purified bradykinin-potentiating pentapeptide (BPP_{5a}) because of the high yields of the coupling and deprotection reactions, accompanied by minimal side reactions, and the need for only one simple final purification step.

The pentapeptide L-pyroglutamyl-L-lysyl-L-tryptophyl-L-alanyl-L-proline (XIV) has been shown to inhibit the conversion of angiotensin I to angiotensin II and the pulmonary inactivation of bradykinin[2,3]. It was first isolated from Bothrops Jararaca venom[4] and has been characterized and synthesized by a solid-phase procedure[3].

While the solid-phase approach will suffice to provide small quantities of peptide, a classical technique has the advantage of being able to yield gram quantities conveniently and reproducibly. In this paper, a classical and economically-viable, industrial procedure is reported for the synthesis, in high yield, of pure bradykinin-potentiating pentapeptide. In most cases, the intermediate peptides

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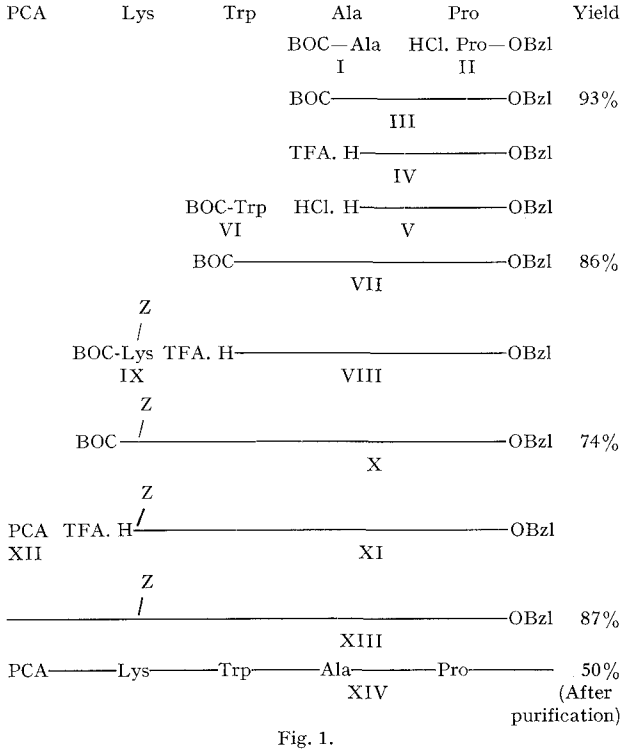

Fig. 1.

were used in the crude state for further reactions, and the t-butyloxycarbonyl (BOC) group was removed by a 50% solution of redistilled trifluoroacetic acid (TFA) in methylene chloride at room temperature. Mixed carbonic anhydrides, used as acylating agents in some of the coupling reactions, were generated in situ with *N*-methylamorpholine and isobutyl chloroformate in tetrahydrofuran or dimethylformamide at −15 °C. *N*-Methylmorpholine was also employed to liberate free amino compounds from their hydrochloride or trifluoro-acetate salts. All reaction afforded at least 10 g of product.

An outline of the original synthesis is shown in Figure 1. First, BOC-L-alanine[5] (I) was coupled to L-proline benzyl ester, liberated from its hydrochloride[6] (II), by a mixed carbonic anhydride reaction. After removal of the BOC group from BOC-L-alanyl-L-proline benzyl ester (III), the oily dipeptide benzyl ester trifluoroacetate (IV) was converted to the corresponding crystalline hydrochloride (V). This enabled small quantities of fully protected dipeptide, present as a contaminant in the trifluoroacetate, to be removed. Next, BOC-L-tryptophan[5] (VI) was coupled to L-alanyl-L-proline benzyl ester using 1-ethyl 3-(3-dimethylaminopropyl) carbodiimide hydrochloride. The BOC group of the resulting protected tripeptide (VII) was removed and, after neutralization, the tripeptide benzyl ester trifluoro-acetate (VIII) was coupled with N_α-BOC-N_ε-benzyloxycarbonyl-L-lysine[5] (IX) by a mixed carbonic anhydride reaction. The BOC group of the protected tetrapeptide (X) afforded by this method was removed and the resulting trifluoro-acetate (XI), after neutralization, was coupled to L-pyroglutamic acid (XII), again by a mixed carbonic anhydride reaction. The product, the fully protected pentapeptide (XIII), was subjected to hydrogenolysis in a 50% solution of glacial acetic acid in methanol, using 10% palladium on charcoal as catalyst, to give the free pentapeptide (XIV).

Some minor difficulties were experienced because of a small amount of cleavage of the N_ε-benzyloxycarbonyl group from lysine during the removal of the N_α-BOC group from the protected tetrapeptide (X). The synthetic scheme was therefore modified as outlined in Figure 2. The tripeptide trifluoro-acetate (VIII), after neutralization, was allowed to react with N_α-benzyloxycarbonyl-N_ε-BOC-L-lysine *p*-nitrophenyl ester[7] (XV) to yield the protected tetrapeptide (XVI). This, in turn, was subjected to catalytic hydrogenolysis in the same manner as the above protected pentapeptide (XIII). The product, N_ε-BOC-L-lysyl-L-tryptophyl-L-alanyl-L-proline (XVII) was acylated with oily *p*-nitrophenyl L-pyroglutamate (XVIII). The BOC group of the resulting pentapeptide (XIX) was removed with a 50% solution of trifluoroacetic acid in methylene chloride containing 1% β-mercaptoethanol.

The crude pentapeptide, L-pyroglutamyl-L-lysyl-L-tryptophyl-L-alanyl-L-proline (XIV), from both syntheses, was purified by column chromatography on Dowex 50 × 2 resin eluted at 38 °C with a gradient of from 0.2 *M* pyridine in acetic acid (pH 3.1) to 2.0 *M* pyridine in acetic acid (pH 5.0) as described by FERREIRA et al.[4].

The bradykinin potentiating pentapeptide (XIV) obtained in this manner was homogeneous when subjected to thin layer chromatography on Brinkmann-EM silica gel G plates when eluted with *n*-butanol, pyridine, acetic acid, water, 30:6:24:20 (Rf 0.78) and isopropanol, ammonium hydroxide 7:3 (Rf 0.33). It was also homogeneous after paper electrophoresis as described by FERREIRA et al.[4]. The following amino-acid analysis was obtained: Glu, 1.03; Lys, 1.00; Trp, 0.96; Ala, 0.98; Pro, 0.98. The synthetic pentapeptide was equipotent with the natural material in the guinea-pig ileum strip assay[2–4].

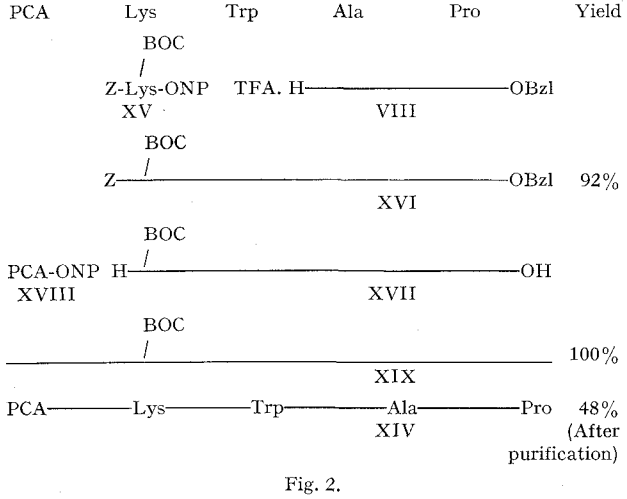

Fig. 2.

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