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Studies on Peptides. CVI.[1,2] Synthesis of a Physalaemin-like Peptide, [Lys^5, Thr^6]-Physalaemin, isolated from the Skin of a Frog, *Uperoleia rugosa*

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An undecapeptide amide, H–Pyr–Ala–Asp–Pro–Lys–Thr–Phe–Tyr–Gly–Leu–Met–NH_2, corresponding to the entire amino acid sequence of an amphibian peptide, [Lys^5, Thr^6]-physalaemin, was synthesized using the thioanisole-mediated trifluoromethanesulfonic acid deprotection procedure. The synthetic peptide was as active as the natural peptide, in terms of the contractility in guinea pig ileum, and its potency relative to that of synthetic substance P was 0.467.

Keywords——amphibian peptide; [Lys^5, Thr^6]-physalaemin; vasoactive polypeptide; physalaemin-like peptide; trifluoromethanesulfonic acid–thioanisole deprotection; contractility in guinea pig ileum

In 1980, as a result of cooperative work between two laboratories, the Department of Medical Pharmacology, University of Rome, headed by Erspamer[3] and the Institute of Pharmaceutical Sciences, Hiroshima University, headed by Nakajima,[4] the chemical natures of two new vasoactive polypeptides from an Australian frog were elucidated.

Methanol extracts of the skin of an Australian leptodactylid frog, *Uperoleia rugosa*, contain several types of biologically active polypeptides. Besides the previously known principles, uperolein[5] (physalaemin-like peptide) and litorin[6] (bombesin-like peptide), two structurally related peptides, [Glu(OEt)^2]-litorin and [Lys^5, Thr^6]-physalaemin, were chemically characterized. It is not known whether the former exists in the tissue or is an artifact produced during isolation. A similar peptide, [Glu(OMe)^2]-litorin,[7] was previously isolated in the methanol extracts of the skin of *Litoria aurea*. In this paper, we wish to report the synthesis of an undecapeptide amide corresponding to the entire amino acid sequence of [Lys^5, Thr^6]-physalaemin.

The synthetic scheme for this peptide is illustrated in Fig. 1. The Z(OMe) group[8] (removable by TFA) was adopted for temporary N$^\alpha$-protection of each fragment. Two functional amino acids in the molecule, Asp and Lys, were protected respectively with Bzl and Z groups, which are both removable by TFMSA.[9,10] In addition, the Met residue was protected as its sulfoxide[11] to prevent partial oxidation during the synthesis and S-alkylation during the deprotection.[12] Three peptide fragments used in this synthesis are known compounds; *i.e.*, Z(OMe)–Leu–Met(O)–NH_2 [1] from our previous synthesis of kassinin,[13] Z(OMe)–Phe–Tyr–Gly–OH [2] from the synthesis of a physalaemin fragment[14] and Z(OMe)–Lys(Z)–Thr–$NHNH_2$ [3] from the synthesis of somatostatin.[15] Thus, the N-terminal protected tetrapeptide, Z–Pyr–Ala–Asp(OBzl)–Pro–OH [4], was newly synthesized starting with H–Pro–OH in a stepwise manner by the active ester procedure, *i.e.*, the Np[16] and Su[17] procedures as shown in Fig. 1.

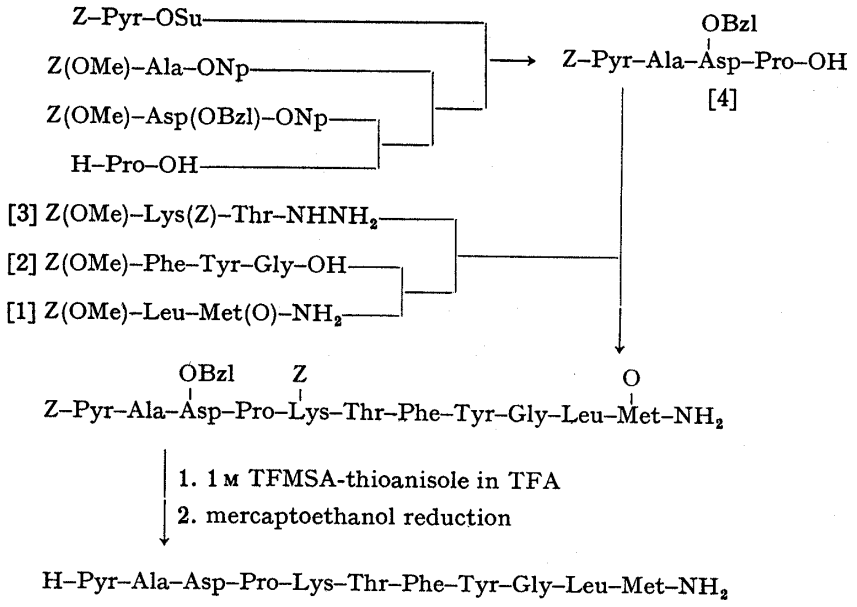

Fig. 1. Synthetic Scheme for [Lys^5, Thr^6]-Physalaemin

The four peptide fragments were then assembled successively to construct the entire peptide backbone. Two fragments, [2] and [4], possessing Gly or Pro as the C-terminus, were condensed by the DCC–HOBT procedure[18] respectively without risk of racemization and the fragment [3] was introduced by the azide procedure.[19] The protected intermediates were purified by repeated precipitation from DMF with methanol or ethanol and the final protected undecapeptide amide by gel-filtration on Sephadex LH-20.

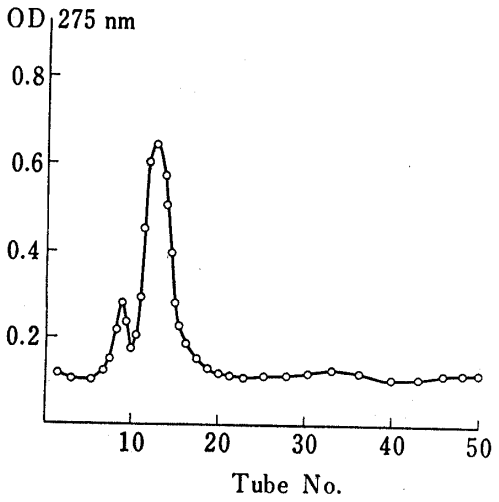

Fig. 2. Partition Chromatography of Synthetic [Lys^5, Thr^6]-Physalaemin on Sephadex G-25

For deprotection, 1 M TFMSA-thioanisole in TFA[10] was employed and *m*-cresol was used as an additional cation scavenger. The deprotected peptide, after conversion to the corresponding acetate by Amberlite CG-4B treatment, was incubated with mercaptoethanol to reduce the Met(O) residue.[11] The reduced product was purified by gel-filtration on Sephadex G-25 followed by partition chromatography according to Yamashiro[20] (Fig. 2). The product thus obtained exhibited a sharp single spot on TLC in two different systems and its purity was confirmed by HPLC, amino acid analysis after acid hydrolysis, and elemental analysis.

The synthetic peptide exhibited *Rf* values identical with those of the natural peptide on high performance thin layer chromatography (HPTLC Merck). We also confirmed that our synthetic peptide was as active as the natural peptide, in terms of the contractility in guinea pig ileum; its potency relative to that of synthetic substance P was 0.467.

Experimental

General experimental methods employed in this investigation are essentially the same as described in Part 88 of this series.[21] *Rf* values in TLC performed on silica gel (Kieselgel G, Merck) refer to the following solvent systems: Rf_1 $CHCl_3$–MeOH–H_2O (8:3:1), Rf_2 *n*-BuOH–AcOH–pyridine–H_2O (30:6:20:24), Rf_3

n-BuOH–AcOH–pyridine–H_2O (4: 1: 1: 2). HPLC was conducted with a Waters compact model, using a μBondapak C_{18} (0.25″×1′) column, and a solvent system of 10 mM $AcONH_4$–acetonitrile (60: 40).

Z(OMe)-Asp(OBzl)-Pro-OH——A mixture of Z(OMe)–Asp(OBzl)–ONp (6.26 g, 12.31 mmol) in THF and H–Pro–OH (2.83 g, 24.62 mmol) in H_2O (25 ml) containing Et_3N (3.4 ml, 24.62 mmol) was stirred at room temperature for 8 h, then the solvent was removed by evaporation and the residue was dissolved in 5% Na_2CO_3. The aqueous phase, after being washed with AcOEt, was acidified with citric acid and the resulting oily precipitate was extracted with AcOEt. The organic phase was washed with 5% citric acid and H_2O–NaCl, dried over Na_2SO_4, and concentrated to dryness. Attempts to crystallize the oily residue were unsuccessful; yield 4.90 g (86%), Rf_1 0.72.

Z(OMe)-Ala-Asp(OBzl)-Pro-OH——Z(OMe)–Asp(OBzl)–Pro–OH (5.97 g, 12.3 mmol) was treated with TFA–anisole (15 ml–6.8 ml) in an ice-bath for 45 min, then dry ether was added. The resulting powder was dried over KOH pellets *in vacuo* for 3 h and dissolved in DMF (20 ml) containing Et_3N (3.4 ml, 24.6 mmol). After addition of Z(OMe)–Ala–ONp (4.62 g, 12.3 mmol) and *N*-methylmorpholine (1.34 ml, 12.3 mmol), the mixture was stirred at 4°C for 12 h. The solvent was evaporated off and the residue was dissolved in 5% Na_2CO_3. The aqueous phase, after being washed with AcOEt, was acidified with citric acid and the resulting oily precipitate was extracted with AcOEt. The organic phase was washed with 5% citric acid and H_2O–NaCl, dried over Na_2SO_4 and concentrated. The residue was triturated with *n*-hexane and recrystallized from AcOEt and *n*-hexane; yield 4.23 g (61%), mp 88—90°C, $[\alpha]_D^{18}$ −59.2° (c=1.0, MeOH). Rf_1 0.46. Amino acid ratios in a 6 N HCl hydrolysate: Ala 1.02, Asp 1.00, Pro 1.02 (recovery of Asp 86%). *Anal.* Calcd for $C_{28}H_{33}N_3O_9 \cdot H_2O$: C, 58.63; H, 6.15; N, 7.33. Found: C, 58.73; H, 5.91; N, 7.30.

Z-Pyr-Ala-Asp(OBzl)-Pro-OH [4]——Z(OMe)–Ala–Asp(OBzl)–Pro–OH (3.54 g, 6.37 mmol) was treated with TFA–anisole (13.8 ml–3.4 ml) as stated above, then *n*-hexane was added. The oily residue was washed with ether, dried over KOH pellets *in vacuo* for 4 h and dissolved in DMF (15 ml) containing Et_3N (1.76 ml, 12.74 mmol). Z–Pyr–OSu (2.30 g, 6.37 mmol) and *N*-methylmorpholine (0.64 ml, 6.37 mmol) were added and the mixture, after being stirred at 4°C for 24 h, was concentrated. The product was purified as stated above by the extraction procedure and recrystallized from AcOEt and ether; yield 3.05 g (75%), mp 85—88°C, $[\alpha]_D^{18}$ −95.6° (c=1.0, MeOH), Rf_1 0.46. Amino acid ratios in a 6 N HCl hydrolysate: Glu 1.04, Ala 0.97, Asp 1.00, Pro 1.15 (recovery of Asp 89%). *Anal.* Calcd for $C_{32}H_{35}N_4O_9 \cdot 2H_2O$: C, 58.62; H, 5.99; N, 8.55. Found: C, 58.28; H, 5.79; N, 8.60.

Z(OMe)-Phe-Tyr-Gly-Leu-Met(O)-NH_2——Z(OMe)–Leu–Met(O)–NH_2 [1] (9.98 g, 23 mmol) was treated with TFA–anisole (36 ml–9 ml) in an ice-bath for 45 min, then dry ether was added. The resulting powder was collected by filtration, then dissolved in 3.6 N HCl–dioxane (7 ml, 1.2 eq) and excess dioxane was removed by evaporation *in vacuo*. The residue was dried over KOH pellets *in vacuo* for 2 h and dissolved in DMF (30 ml) together with Et_3N (3.86 ml, 28 mmol), Z(OMe)–Phe–Tyr–Gly–OH [2] (15.39 g, 28 mmol) and HOBT (5.21 g, 34 mmol). DCC (7.01 g, 34 mmol) was added to this stirred mixture, and after 12 h at room temperature the solution was filtered. The filtrate was concentrated and the residue was treated with 5% Na_2CO_3. The resulting powder was washed with 5% Na_2CO_3, 5% citric acid and H_2O and precipitated twice from DMF with EtOH; yield 14.24 g (77%), mp 190—193°C, $[\alpha]_D^{20}$ −45.6° (c=1.0, DMF). Rf_1 0.52. Amino acid ratios in a 6 N HCl hydrolysate (with phenol): Phe 0.98, Tyr 0.96, Gly 0.98, Leu 1.00, Met+Met(O), 0.80 (recovery of Leu 87%). *Anal.* Calcd for $C_{40}H_{52}N_6O_{10}S \cdot 2H_2O$: C, 59.72; H, 6.46; N, 10.10. Found: C, 60.09; H, 6.55; N, 10.00.

Z(OMe)-Lys(Z)-Thr-Phe-Tyr-Gly-Leu-Met(O)-NH_2——The above protected pentapeptide amide (3.91 g, 4.82 mmol) was treated with TFA–anisole (20 ml–4 ml) as stated above, then dry ether was added. The resulting powder was collected by filtration, dried over KOH pellets *in vacuo* for 3 h and dissolved in DMF (10 ml) containing Et_3N (0.67 ml, 4.82 mmol). The azide [prepared from 4.05 g (7.23 mmol) of Z(OMe)–Lys(Z)–Thr–$NHNH_2$] in DMF (10 ml) and Et_3N (1.1 ml, 7.96 mmol) were added to the above ice-chilled solution and the mixture was stirred at 4°C for 24 h. When the ninhydrin test became negative, the solvent was evaporated off and the residue was treated with 5% citric acid. The resulting powder was washed with 5% citric acid and H_2O and then precipitated twice from DMF with MeOH; yield 2.37 g (42%), mp 212—213°C, $[\alpha]_D^{20}$ −27.9° (c=0.8, DMF). Rf_1 0.59. Amino acid ratios in a 6 N HCl hydrolysate (with phenol): Lys 1.10, Thr 1.03, Phe 0.98, Tyr 0.96, Gly 0.96, Leu 1.00, Met+Met(O) 0.80 (recovery of Leu 81%). *Anal.* Calcd for $C_{58}H_{77}N_9O_{15}S \cdot H_2O$: C, 58.52; H, 6.61; N, 10.59. Found: C, 58.56; H, 6.71; N, 10.11.

Z-Pyr-Ala-Asp(OBzl)-Pro-Lys(Z)-Thr-Phe-Tyr-Gly-Leu-Met(O)-NH_2——The above protected heptapeptide amide (0.61 g, 0.52 mmol) was treated with TFA–anisole (1.1 ml–0.2 ml) and the deprotected peptide was converted to the corresponding hydrochloride with 3.6 N HCl–dioxane (0.29 ml, 1.04 mmol) as described above. Trituration of the hydrochloride with ether afforded a powder, which was dried over KOH pellets *in vacuo* for 4 h and dissolved in DMF (7 ml) together with Et_3N (0.07 ml, 0.52 mmol), HOBT (0.14 g, 0.94 mmol) and Z–Pyr–Ala–Asp(OBzl)–Pro–OH (0.48 g, 0.78 mmol). DCC (0.19 g, 0.94 mmol) and *N*-methylmorpholine (0.09 ml, 0.78 mmol) were further added and the mixture was stirred at 4°C for 24 h, then filtered. The filtrate was concentrated and the residue was treated with 5% citric acid and ether. The resulting powder was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O. A half of the product (308 mg) was dissolved in a small amount of DMF and the solution was applied to a column of Sephadex LH-20 (3×140 cm), which was eluted with the same solvent. The UV absorption at 275 nm was determined in each fraction (8 ml).

The main fractions (tube Nos. 92—98) were combined, the solvent was evaporated off, and the residue was treated with AcOEt to afford a powder. The rest of the crude product was similarly purified; total yield 585 mg (69%), mp 159—161°C, $[\alpha]_D^{18}$ −51.0° (c=0.6, DMF), Rf_1 0.74. Amino acid ratios in a 6 N HCl hydrolysate (with phenol): Glu 1.08, Ala 0.98, Asp 1.02, Pro 0.96, Lys 0.99, Thr 0.99, Phe 0.97, Tyr 0.95, Gly 1.00, Leu 1.00, Met+Met(O) 0.73 (recovery of Leu 79%). *Anal.* Calcd for $C_{81}H_{103}N_{13}O_{21}S \cdot 3H_2O$: C, 57.88; H, 6.54; N, 10.83. Found: C, 57.71; H, 6.40; N, 11.12.

H-Pyr-Ala-Asp-Pro-Lys-Thr-Phe-Tyr-Gly-Leu-Met-NH_2——The above protected undecapeptide amide (83 mg, 51 μmol) was treated with 1 M TFMSA–thioanisole in TFA (3.06 ml, 60 eq) in the presence of *m*-cresol (0.16 ml, 30 eq) in an ice-bath for 2 h, then dry ether was added. The resulting powder was collected by filtration and dissolved in a small amount of H_2O. The solution was treated with Amberlite CG-4B (acetate form, approximately 1 g) in an ice-bath for 45 min, then filtered. The pH of the filtrate was adjusted to 10 with 5% NH_4OH and after 10 min, to 6 with 3% AcOH. The solution was lyophilized and the residue was dissolved in H_2O (50 ml). The solution was incubated with mercaptoethanol (0.4 ml, 100 eq) at 40°C for 8 h, while the progress of reduction of the Met(O) residue was monitored by TLC. The solution was then applied to a column of Sephadex G-25 (1.8×145 cm), which was eluted with 0.1 N AcOH, and the UV absorption at 275 nm was determined in each fraction (2 ml). The main fractions (tube Nos. 152—160) were combined and the solvent was removed by lyophilization to give a crude product as a fluffy powder; yield 44 mg (69%).

The crude product was dissolved in a small amount of the upper phase of *n*-BuOH–AcOH–H_2O (4: 1: 5) and the solution was applied to a column of Sephadex G-25 (1.8×145 cm), which had previously been equilibrated with the lower phase of the above solvent system. The column was then eluted with the upper phase and individual fractions (3 ml each) were collected. The UV absorption at 275 nm was determined (Fig. 2). The main fractions (tube Nos. 10—21) were combined, the solvent was removed by evaporation and the residue was lyophilized from 0.1 N AcOH to give a fluffy white powder; yield 35 mg (55%). $[\alpha]_D^{20}$ −88.2° (c=0.5, H_2O), Rf_2 0.66, Rf_3 0.56. Amino acid ratios in 6 N HCl hydrolysate: Glu 1.10, Ala 1.04, Asp 0.99, Pro 1.04, Lys 1.01, Thr 1.01, Phe 1.01, Tyr 1.00, Gly 1.04, Leu 1.00, Met 0.82 (recovery of Leu 79%). *Anal.* Calcd for $C_{58}H_{84}N_{13}O_{16}S \cdot CH_3COOH \cdot 4H_2O$: C, 52.08; H, 6.99; N, 13.16. Found: C, 52.02; H, 6.69; N, 12.97.

The synthetic peptide, thus obtained, exhibited a single peak on HPLC at a retention time of 4.03 min (flow rate=1 ml/min), under the conditions described in the general procedure.

Identity of the Synthetic Peptide with the Native Peptide——A comparison of the synthetic peptide with the native peptide isolated from the skin of *Uperoleia rugosa* was carried out by high performance thin layer chromatography (HPTLC Merck) and the chromatogram was stained with fluorescamine. The synthetic and the native undecapeptide amides showed the same chromatographic properties with two solvent systems: *Rf* 0.26 [*n*-BuOH–AcOH–H_2O (4: 1: 1)] and *Rf* 0.53 [*n*-BuOH–AcOH–pyridine–H_2O (4: 1: 1: 1)].

The synthetic peptide was as active as the natural peptide in terms of the contractility in guinea pig ileum, and its potency relative to that of synthetic substance P was 0.467.

References and Notes

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2) Amino acids, peptides and their derivatives mentioned in this investigation are of the L-configuration. The following abbreviations are used: Z=benzyloxycarbonyl, Z(OMe)=*p*-methoxybenzyloxycarbonyl, Bzl=benzyl, Np=*p*-nitrophenyl, Su=*N*-hydroxysuccinimide, DCC=dicyclohexylcarbodiimide, HOBT=*N*-hydroxybenzotriazole, TFA=trifluoroacetic acid, DMF=dimethylformamide, TFMSA=trifluoromethanesulfonic acid, THF=tetrahydrofuran, HPLC=high performance liquid chromatography.
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