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Studies on Peptides. CXIV.[1,2] Synthesis of Hylambates-Kassinin, a Frog Skin Tachykinin Peptide

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Hylambates-kassinin, a new amphibian skin dodecapeptide of the tachykinin family, was synthesized in a conventional manner using thioanisole-mediated deprotection with trifluoromethanesulfonic acid. The contractile potency of synthetic hylambates-kassinin in isolated guinea-pig duodenum was 1.4 times higher than that of synthetic kassinin.

Keywords——synthesis of hylambates-kassinin; [Glu^2, Pro^5]-kassinin; amphibian peptide; tachykinin peptide; thioanisole-mediated deprotection; contractile activity

Recently, Yasuhara *et al.*[3] elucidated the structures of two new dodecapeptides of the tachykinin family,[4] which were isolated from methanol extracts of the skin of an African rhacophorid frog (*Hylambates maculatus*). The structure of the one named hylambates-kassinin differs from that of kassinin[5] at two positions, 2 and 5, *i.e.*, Val^2 and Ser^5 in kassinin were replaced by Glu^2 and Pro^5, respectively, in hylambates-kassinin as shown in Fig. 1. The other peptide, named hylambatin, is a homologous peptide to the former, but has a characteristic C-terminal dipeptide unit, Met–Met–NH_2, instead of Leu–Met–NH_2 of tachykinin peptides.

Subsequent to our synthesis of kassinin,[6] we wish to report in this paper the synthesis of hylambates-kassinin. The synthetic scheme for this new amphibian skin peptide, [Glu^2, Pro^5]-kassinin, is illustrated in Fig. 2; our newly devised TFMSA deprotecting procedure[7] was employed.

In our previous synthesis of kassinin, the Met residue was protected as its sulfoxide.[8] However, the present synthesis was carried out without protection of its thio-ether linkage, since the *S*-alkylation[9] observable during the TFA deprotection of N^{α}-protecting groups, such as the Boc or the Z(OMe) groups, can be minimized by the use of anisole containing EDT, and *S*-alkyl compounds, if formed, can be reduced easily by thiols at the last stage of the synthesis.[10] Thus, H–Leu–Met–NH_2, derived from the Boc-derivative[11] by treatment with TFA in the presence of anisole containing EDT, was condensed, *via* the azide,[12] with Boc–Phe–Val–Gly–$NHNH_2$, prepared by the azide coupling of the known dipeptide hydrazide, Boc–Phe–Val–$NHNH_2$,[13] and H–Gly–OMe, followed by usual hydrazine treatment. Then the peptide chain of the resulting pentapeptide amide, Boc–Phe–Val–Gly–Leu–Met–NH_2,

Hylambates-kassinin	H–Asp–Glu–Pro–Lys–Pro–Asp–Gln–Phe–Val–Gly–Leu–Met–NH_2
Hylambatin	H–Asp–Pro–Pro–Asp–Pro–Asp–Arg–Phe–Tyr–Gly–Met–Met–NH_2
Kassinin	H–Asp–Val–Pro–Lys–Ser–Asp–Gln–Phe–Val–Gly–Leu–Met–NH_2

Fig. 1. Structure of Hylambates-Kassinin and Related Peptides

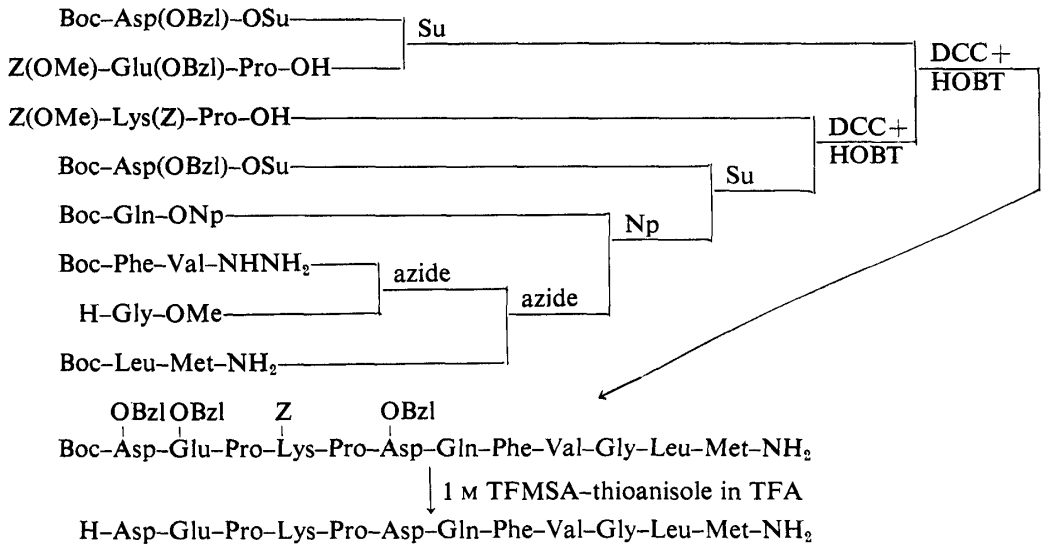

Fig. 2. Synthesis of Hylambates-Kassinin

was elongated by stepwise additions of two amino acids residues, Boc–Gln–OH and Boc–Asp(OBzl)–OH, *via* the active esters,[14] such as Np[14] or Su[15] esters, followed by the DCC plus HOBT condensations[16] of two fragments, Z(OMe)–Lys(Z)–Pro–OH[17] and Boc–Asp(OBzl)–Glu(OBzl)–Pro–OH. The latter tripeptide was prepared by condensation of Boc–Asp(OBzl)–OH with a TFA-treated sample of the known dipeptide, Z(OMe)–Glu(OBzl)–Pro–OH[18] *via* the Su ester. Throughout this synthesis, every intermediate was purified by precipitation from DMF or DMSO with methanol or ethanol.

The protected hylambates-kassinin was treated with 1 M TFMSA in TFA to remove all protecting groups. Thioanisole and *m*-cresol were used as cation scavengers. The former has an ability to accelerate the acid deprotection,[19] and the latter to trap efficiently alkyl cations derived from the protecting groups.[20] This treatment was repeated once more to ensure complete deprotection. The deprotected peptide was incubated with DTT[10] to reduce the sulfoxide and the *S*-alkyl derivative, probably formed during manipulation, and then purified by gel-filtration on Sephadex LH-20, using 90% aqueous methanol as an eluant, to give a homogeneous product on thin layer chromatography (TLC). The product was not freely soluble in water, so the above purification procedure was judged to be a suitable method.

When contractile response in isolated guinea-pig duodenum[21] was compared with that of synthetic kassinin (taken as 1),[6] the relative potency of synthetic hylambates-kassinin was 1.44.

Experimental

General experimental methods employed in this paper are essentially the same as described in Part LXXXVIII[22] of this series. *Rf* values in TLC 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 (4:1:1:2), Rf_3 *n*-BuOH–AcOH–pyridine–H_2O (30:6:20:24).

Boc–Phe–Val–Gly–OMe——The azide [prepared from 5.00 g (13.2 mmol) of Boc–Phe–Val–$NHNH_2$[13]] in DMF (30 ml) and Et_3N (1.8 ml, 13.2 mmol) were added to an ice-chilled solution of H–Gly–OMe [prepared from the corresponding hydrochloride (2.49 g, 19.8 mmol)] in DMF (20 ml) and the mixture was stirred at 4 °C for 24 h. 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 precipitated from DMF with ether; yield 3.79 g (66%), mp 163—167 °C, $[\alpha]_D^{16}$ +2.2° (c=0.5, DMF), Rf_1 0.78. *Anal.* Calcd for $C_{22}H_{33}N_3O_6 \cdot 1/2H_2O$: C, 59.44; H, 7.71; N, 9.45. Found: C, 58.96; H, 7.85; N, 9.54.

Boc–Phe–Val–Gly–$NHNH_2$——The above protected tripeptide ester (3.78 g, 8.7 mmol) in DMF–MeOH (1:1, 40 ml) was treated with hydrazine hydrate (2.6 ml, 5 eq) overnight and the resulting solid was precipitated from DMF

with MeOH; yield 3.02 g (80%), mp 158—161 °C, $[\alpha]_D^{16} -10.3°$ ($c=1.0$, DMF), Rf_1 0.53. *Anal.* Calcd for $C_{21}H_{33}H_5O_5 \cdot 1/2H_2O$: C, 56.73, H, 7.71; N, 15.76. Found: C, 56.84; H, 7.88; N, 15.85.

Boc–Phe–Val–Gly–Leu–Met–NH_2——Boc–Leu–Met–NH_2 (2.49 g, 6.9 mmol) was treated with TFA–anisole containing 2% EDT (7.5–2.7 ml) in an ice-bath for 90 min, then dry ether was added. The resulting powder was dried over KOH pellets *in vacuo* for 3 h and dissolved in DMF (15 ml) containing Et_3N (1.1 ml, 7.6 mmol). The azide [prepared from 3.0 g (6.9 mmol) of Boc–Phe–Val–Gly–$NHNH_2$] in DMF (15 ml) and Et_3N (1.0 ml, 6.9 mmol) were added to the above ice-chilled solution and the mixture was stirred at 4 °C for 24 h. The solvent was evaporated off and the residue was treated with 5% citric acid to afford a powder, which was washed with 5% citric acid and H_2O and precipitated from DMF with MeOH; yield 3.01 g (66%), mp 218—222 °C, $[\alpha]_D^{16} +3.1°$ ($c=1.0$, DMF), Rf_1 0.64. Amino acid ratios in 6 N HCl hydrolysate; Phe 0.91, Val 0.96, Gly 1.03, Leu 1.00, Met 0.78 (recovery of Leu 86.2%). *Anal.* Calcd for $C_{32}H_{52}N_6O_7S$: C, 57.81; H, 7.88; N, 12.64. Found: C, 57.60; H, 7.92; N, 12.66.

Boc–Gln–Phe–Val–Gly–Leu–Met–NH_2——Boc–Phe–Val–Gly–Leu–Met–NH_2 (3.0 g, 4.5 mmol) was treated with TFA–anisole containing 2% EDT (9–3.4 ml) and the N^{α}-deprotected peptide isolated as stated above was dissolved in DMF (30 ml), together with Et_3N (1.3 ml, 9.0 mmol) and Boc–Gln–ONP (3.06 g, 8.3 mmol). After being stirred overnight, the solution was concentrated and the residue was treated with 5% citric acid. The resulting powder was washed with 5% $NaHCO_3$, 5% citric acid and H_2O and precipitated from DMSO with MeOH; yield 2.76 g (77%), mp 241—245 °C, $[\alpha]_D^{16} -22.8°$ ($c=1.0$, DMSO), Rf_1 0.60. Amino acid ratios in 6 N HCl hydrolysate: Glu 1.05, Phe 0.89, Val 0.97, Gly 1.02, Leu 1.00, Met 0.83 (recovery of Leu 86.9%). *Anal.* Calcd for $C_{37}H_{60}N_8O_9S \cdot H_2O$: C, 54.79; H, 7.71: N, 13.82. Found: C, 54.51; H, 7.71; N, 13.74.

Boc–Asp(OBzl)–Gln–Phe–Val–Gly–Leu–Met–NH_2——The above protected hexapeptide amide (2.70 g, 3.4 mmol) was treated with TFA–anisole containing 2% EDT (8–3.0 ml) and the N^{α}-deprotected peptide isolated as stated above was dissolved in DMSO–DMF (2 : 1, 80 ml) together with Et_3N (0.7 ml, 5.1 mmol) and Boc–Asp(OBzl)–OSu (2.86 g, 6.8 mmol). After being stirred overnight, the solution was concentrated and the residue was treated with 5% citric acid. The resulting powder was washed as stated above and precipitated from DMSO with MeOH; yield 3.0 g (88%), mp 218—221 °C, $[\alpha]_D^{16} -13.3°$ ($c=1.0$, DMSO), Rf_1 0.55. Amino acid ratios in 6 N HCl hydrolysate; Asp 0.98, Glu 1.08, Phe 0.89, Val 0.97, Gly 1.03, Leu 1.00, Met 0.79 (recovery of Leu 84.4%). *Anal.* Calcd for $C_{48}H_{71}N_9O_{12}S \cdot H_2O$: C, 56.73; H, 7.24; N, 12.41. Found: C, 56.48; H, 7.13; N, 12.62.

Z(OMe)–Lys(Z)–Pro–Asp(OBzl)–Gln–Phe–Val–Gly–Leu–Met–NH_2——The above protected heptapeptide amide (2.70 g, 2.7 mmol) was treated with TFA–anisole containing 2% EDT (8–3.0 ml) and the N^{α}-deprotected peptide isolated as stated above was dissolved in 4.5 N HCl–dioxane. The solution was stirred for 15 min, then dry ether was added. The resulting power was dried over KOH pellets *in vacuo* for 3 h and dissolved in DMSO–DMF (2 : 1, 50 ml), together with Et_3N (0.4 ml, 3.0 mmol), Z(OMe)–Lys(Z)–Pro–OH (2.92 g, 5.4 mmol) and HOBT (0.73 g, 5.4 mmol). After addition of DCC (1.23 g, 5.9 mmol), the solution was stirred overnight, then filtered and concentrated. Treatment of the residue with 5% citric acid afforded a powder, which was washed as stated above and precipitated from DMSO with MeOH; yield 2.33 g (60%), mp 207—210 °C, $[\alpha]_D^{16} -19.9°$ ($c=1.0$, DMSO), Rf_1 0.63. Amino acid ratios in 6 N HCl hydrolysate: Lys 0.94, Pro 0.98, Asp 1.09, Glu 1.02, Phe 0.88, Val 0.98, Gly 1.05, Leu 1.00, Met 0.78 (recovery of Leu 79.4%). *Anal.* Calcd for $C_{71}H_{96}N_{12}O_{17}S \cdot 3H_2O$: C, 57.78; H, 6.97; N, 11.39. Found: C, 57.63; H, 6.70; N, 11.38.

Boc–Asp(OBzl)–Glu(OBzl)–Pro–OH——Z(OMe)–Glu(OBzl)–Pro–OH (4.0 g, 8.0 mmol) was treated with TFA–anisole (8–2 ml) as usual, then *n*-hexane was added. The resulting oil was dried over KOH pellets *in vacuo* for 3 h and dissolved in DMF (30 ml), together with Et_3N (2.5 ml, 17.6 mmol) and Boc–Asp(OBzl)–OSu (3.37 g, 8.0 mmol). After being stirred overnight, the solution was concentrated and the residue, after treatment with 5% citric acid, was dissolved in AcOEt. The organic phase was washed with 5% citric acid and H_2O–NaCl, dried over Na_2SO_4 and concentrated. The residue was purified by column chromatography on silica gel, which was eluted with $CHCl_3$–MeOH (10 : 0.5). Fractions containing the substance of Rf_1 0.67 were combined and the solvent was removed by evaporation to give an oily residue; yield 4.0 g (78%). Amino acid ratios in 6 N HCl hydrolysate: Asp 1.06, Glu 1.01, Pro 1.00.

Boc–Asp(OBzl)–Glu(OBzl)–Pro–Lys(Z)–Pro–Asp(OBzl)–Gln–Phe–Val–Gly–Leu–Met–NH_2——The above protected nonapeptide amide (1.50 g, 1.1 mmol) was treated with TFA–anisole containing 2% EDT (4.5–1.6 ml) and the N^{α}-deprotected peptide isolated as stated above was dissolved in 4.45 N HCl–dioxane. After 15 min, dry ether was added. The resulting powder was collected by filtration, dried over KOH pellets *in vacuo* for 3 h and dissolved in DMSO–DMF (2 : 1, 10 ml), together with Et_3N (0.2 ml, 1.2 mmol), Boc–Asp(OBzl)–Glu(OBzl)–Pro–OH (1.36 g, 2.1 mmol) and HOBT (0.29 g, 2.1 mmol). After addition of DCC (0.48 g, 2.3 mmol), the solution was stirred overnight, filtered and concentrated. Treatment of the residue with 5% citric aid afforded a powder which was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O and precipitated from DMSO with EtOH; yield 1.30 g (65%), mp 217—220 °C, $[\alpha]_D^{16} -30.9°$ ($c=0.8$, DMSO), Rf_1 0.63. Amino acid ratios in 6 N HCl hydrolysate: Asp 1.96, Glu 1.97, Pro 1.92, Lys 1.01, Phe 0.88, Val 0.92, Gly 1.08, Leu 1.00, Met 0.78 (recovery of Leu 86.3%). *Anal.* Calcd for $C_{95}H_{127}N_{15}O_{23}S \cdot 7H_2O$: C, 56.90; H, 7.09; N, 10.48. Found: C, 57.03; H, 6.82; N, 10.28.

H–Asp–Glu–Pro–Lys–Pro–Asp–Gln–Phe–Val–Gly–Leu–Met–NH_2——The above protected dodecapeptide amide (200 mg, 0.11 mmol) was treated with 1 M TFMSA–thioanisole in TFA (5.3 ml) in the presence of *m*-cresol

(0.6 ml, 50 eq) in an ice-bath for 90 min, then dry ether was added and the resulting powder was collected by centrifugation. This treatment was repeated once more under identical conditions. The deprotected peptide dissolved in H_2O (5 ml) was treated with Amberlite CG-4B (acetate form, approximately 1 g) for 30 min. The resin was removed by filtration and the filtrate was lyophilized. The residue dissolved in H_2O (4 ml) was next incubated with DTT (327 mg, 20 eq) at 37 °C for 2 d and the solution was applied to a column of Sephadex LH-20 (3.5 × 140 cm), which was eluted with 90% MeOH. Individual fractions (10 ml each) were collected and an aliquot of each was subjected to the ninhydrin text. Fractions corresponding to the main peak (tube Nos. 37—43) were combined, the solvent was removed by evaporation and the residue was lyophilized to give a white fluffy powder; yield 49.6 mg (34%) $[\alpha]_D^{16}$ −90.7° (c=0.7, 20% AcOH), Rf_2 0.26, Rf_3 0.33. Amino acid ratios in 6 N HCl hydrolysate: Asp 1.85, Glu 1.94, Pro 1.80, Lys 0.96, Phe 0.89, Val 0.99, Gly 1.01, Leu 1.00, Met 0.83 (recovery of Leu 78%). *Anal.* Calcd for $C_{61}H_{95}N_{15}O_{19}S \cdot CH_3COOH \cdot 4H_2O$: C, 50.22; H, 7.16; N, 13.95. Found: C, 50.32; H, 6.92; N, 13.93.

The synthetic peptide exhibited a single peak (retention time 7.0 min) on HPLC (Waters 204 compact model) using a column of TSK-gel LS 410K (0.4 × 30 cm) by isocratic elution with 30% acetonitrile in 0.25 N triethylammonium phosphate (pH 3.0) at a flow rate of 1.0 ml per min.

References and Notes

1) Part CXIII: *Int. J. Peptide Protein Res.*, "in press."
2) Amino acids, peptides and their derivatives mentioned in this report are of the L-configuration. The following abbreviations are used: Z = benzyloxycarbonyl, Z(OMe) = *p*-methoxybenzyloxycarbonyl, Bzl = benzyl, Boc = *tert*-butoxycarbonyl, TFA = trifluoroacetic acid, TFMSA = trifluoromethanesulfonic acid, DCC = dicyclohexylcarbodiimide, HOBT = *N*-hydroxybenzotriazole, EDT = ethanedithiol, DMF = dimethylformamide, DMSO = dimethylsulfoxide, DTT = dithiothreitol, Np = *p*-nitrophenyl, Su = *N*-hydroxysuccinimide, Tcp = 2,4,5-trichlorophenyl.
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