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Synthesis of the Protected C-Terminal Lys[5]-Heptapeptide* of Eledoisin by the Merrifield Method

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The protected heptapeptide amide Z-Lys(Boc)-Ala-Phe-Ile-Gly-Leu-Met-NH_2 was synthesized by the Merrifield solid-phase method followed by cleavage of the peptide resin with ethanolic ammonia. After purification on silicagel and Sephadex LH-20 the over-all yield was 14 % of theory based on Boc-methionyl resin. Deblocking of the ε-amino group resulted in a significant increase in biological activity.

Ammonolysis by alcoholic ammonia of resin-bound protected peptides with a glycine "anchor" has recently received much attention as a direct method for obtaining the corresponding C-terminal amides.[1–6] To test the application of this method to a peptide with C-terminal methionine, an eledoisin analogue was considered suitable. As the amide configuration is essential for biological activity,[7] a biological assay could thus be included in the analytical characterization of the product. In order to facilitate the purification, a shortened sequence was preferred to eledoisin itself, and, as both N^{α}-*mono*acylated and $N^{\alpha},N^{\varepsilon}$-*di*acylated Lys-Ala-Phe-Ile-Gly-Leu-Met-NH_2 have been synthesized [8] and found biologically active,[9] the synthesis of the corresponding N^{α}-Z,N^{ε}-Boc derivative was undertaken.

The protected heptapeptide resin was synthesized in a stepwise manner according to the procedure of Merrifield,[10] using methylene chloride, ethanol, and acetic acid as solvents, and deblocking twice with N HCl in acetic acid in order to ensure a complete cleavage of the Boc group. In this way Boc-methionyl resin with a mehionine content of 0.6 mequiv. per g was subjected

* Abbreviations follow the rules of the IUPAC–IUB commission on biochemical nomenclature: *J. Biol. Chem.* **241** (1966) 2491.

to six cycles of deblocking, neutralization and coupling. The Boc-amino acids used were prepared according to Schnabel.[11] The excess of Boc-amino acid and carbodiimide employed in the coupling step was for Leu, Ile, and Ala: two-fold, for Gly and Phe: three-fold and for Lys: four-fold. After the coupling of the final residue, the weight of the resin had increased to 68 % of that expected when calculated on the methionine content of the starting material.

To obtain as complete a cleavage from the resin as possible, three methods of ammonolysis were employed successively on the entire quantity of peptide resin, *viz.* treatment with (1) saturated ethanolic ammonia at room temperature, (2) ethanol saturated with ammonia at 0°C employed at room temperature in a sealed flask,[4] and (3) liquid ammonia at atmospheric pressure.[3] After concentration of the filtrates and crystallization from acetic acid/ethanol the three products behaved identically in thin-layer chromatography in three solvent systems. The best separation was obtained in solvent system S1, where three major impurities could be distinguished. By combined use of preparative silicagel and Sephadex LH-20 column fractionation it was possible to obtain the protected peptide in a chromatographically pure state. Elemental analysis indicates crystallization with one molecule of ethanol. The over-all yield was 14 % of theory, based on the quantity of Boc-methionyl resin used. However, in view of the observed formation of by-products during the work-up, it is probable that the yield could be improved if a different purification procedure were employed. For further characterization the N^{ε}-Boc group was removed by trifluoroacetic acid solvolysis. The homogeneity of the ether-precipitated and freeze-dried N^{ε}-trifluoroacetate was controlled by high-voltage paper electrophoresis. Elemental analysis indicates a binding of excess trifluoroacetic acid.

With bradykinin (Sandoz) as reference the biological activity of the N^{α},N^{ε}-*di*acylated and the N^{α}-*mono*acylated peptide amides was compared by the guinea-pig ileum and the rabbit blood pressure methods. The latter compound was found to be the more active, in agreement with the finding of Lübke *et al.*,[9] and the activity was of the same order of magnitude as reported for eledoisin and related compounds.[7,12,13]

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EXPERIMENTAL

Melting points are uncorrected. Ascending thin-layer chromatography was performed on commercial plates (DC-Fertigplatten, Kieselgel F 254, E. Merck AG, Darmstadt). Solvent systems: S1 (chloroform/acetic acid/methanol by volume 90/5/5), S2 (2-butanol/formic acid/water by volume 75/15/10) and S3 (2-butanol/10 % aqueous ammonia by volume 85/15). Chromatograms and electropherogram were developed by spraying with tertiary butyl hypochlorite, followed by *p*-tolidine/potassium iodide. Optical rotations were measured with the Perkin-Elmer model 141 polarimeter. The apparatus used for the manual solid-phase synthesis was similar to the one described by Kusch.[14] The resin (Bio-beads S-X2, 200—400 mesh) was obtained from Bio-Rad Laboratories, Richmond, California, and was chloromethylated according to Merrifield.[15] Methylene chloride (May and Baker Ltd., Dagenham, Essex) was stored over potassium carbonate and

Table 1. Melting point and optical rotation of amino acid derivatives.

Derivative		m.p.°C	$[\alpha]_{578}^{t\,\circ}$	$[\alpha]_{D}^{t\,\circ}$	*c*	solv.	*t*	Literature
Boc-L-methionine [a]		56–7						
»	DCHA-salt	140–1	+15.1		1	DMF	25	
»	»	138–9	+17.3		1	DMF	18–25	Schnabel [11]
Boc-L-leucine		84–6	−25.6	−24.3	1	HOAc	25	
»		78–81	−30.0		1	HOAc	18–25	Schnabel [11]
»		86–7		−24		HOAc		Beyerman *et al.*[3]
Boc-glycine		88–9						
»		94–5						Schnabel [11]
Boc-L-isoleucine		73–4	+ 2.6	+ 2.5	1	HOAc	25	
»		66–8	+ 2.5		1	HOAc	18–25	Schnabel [11]
»		57–9		+ 2.5	1.8	HOAc		Beyerman *et al.*[3]
Boc-L-phenylalanine		86–7	− 4.0	− 4.2	1.5	HOAc	25	
»		84–6	− 4.0		1	HOAc	18–25	Schnabel [11]
Boc-L-alanine		83–4	−25.9	−24.7	1	HOAc	25	
»		80–2	−25.2		1	HOAc	18–25	Schnabel [11]
N^{α}-Z,N^{ε}-Boc-L-lysine		oil	− 3.2		1	HOAc	25	
»		oil	− 3.0		1	HOAc	18–25	Schnabel [11]
»	DCHA-salt	156–7		+ 7.2	1	EtOH	25	
»	»	156–7		+ 7.8	1	EtOH	20	Wünsch *et al.*[16]

[a] Found: C 48.2; H 7.9; N 5.5; S 13.0.
Calc. C 48.2; H 7.7; N 5.6; S 12.9.

DCHA: dicyclohexylamine
HOAc: glacial acetic acid
DMF: dimethylformamide
EtOH: absolute ethanol

distilled before use. Acetic acid ("Pronalys" glacial acetic acid, May and Baker Ltd.) was used as such. Ethanol was commercial absolute ethanol. The Boc-amino acids were prepared according to Schnabel,[11] and their melting points and rotations are given in Table 1 together with those of the literature. Isolated reaction products were dried *in vacuo* over potassium hydroxide pellets at room temperature. Bioassay: *Guinea-pig ileum.* A segment of guinea-pig ileum was suspended in a 2.5 ml bath of Tyrode's solution at 34°C, through which air was bubbled. 10—30 μl of solutions containing 0.5—2.0 μg polypeptide per ml was added to the bath. These solutions were prepared from a solution of 1 mg polypeptide in 200 μl acetic acid by dilution with water. *Rabbit blood pressure.* The blood pressure was measured in the carotic artery of a rabbit under urethane anæsthesia. 0.2—1.0 ml of solutions containing 2—10 μg polypeptide per ml was given in an ear vein. These solutions were prepared from a solution of 1 mg polypeptide in 200 μl acetic acid by dilution with 10 % aqueous dimethylformamide containing 0.8 % sodium chloride.

Boc-L-methionyl resin. Chloromethylated resin (10.0 g, 19 mequiv. of Cl) was heated under reflux with oily Boc-L-methionine (5.0 g, 20 mmole) and triethylamine (3.0 ml, 21 mmole) in ethanol (40 ml) for 40 h. Yield 12.0 g after filtration, washing and drying.

L-Methionyl resin hydrochloride. Boc-L-methionyl resin (6.5 g) was transferred to the reaction vessel,[14] and after washing with two 50 ml portions of acetic acid, the Boc group was cleaved by shaking with two 60 ml portions of N HCl in acetic acid for 5 and 30 min, respectively.

L-Methionyl resin. After washing with three 50 ml portions of acetic acid, three 50 ml portions of ethanol and three 75 ml portions of methylene chloride, the aminoacyl resin hydrochloride was shaken with a solution of triethylamine (5.0 ml, 36 mmole) in methylene chloride (70 ml) for 10 min. The filtrate was combined with those from the three subsequent methylene chloride washings and concentrated to dryness on a rotary evaporator under reduced pressure. By Volhard titration of the residual triethylammonium chloride, the methionine content of the material in the reaction vessel was found to be 4.03 mequiv.

Boc-L-leucyl-L-methionyl resin. After washing with one further portion of methylene chloride, the aminoacyl resin was shaken with Boc-L-leucine (2.8 g, 11 mmoles) in methylene chloride (60 ml.) for 30 min before addition of a solution of dicyclohexylcarbodiimide (2.3 g, 11 mmoles) in methylene chloride (10 ml). The coupling proceeded over night at room temperature. Afterwards, the peptide resin was washed as above with three portions of methylene chloride and three portions of ethanol.

N^{α}-Z,N^{ε}-Boc-L-lysyl-L-alanyl-L-phenylalanyl-L-isoleucyl-glycyl-L-leucyl-L-methionyl resin. The cycle was repeated successively with Boc-glycine (2.8 g, 16 mmoles), Boc-L-isoleucine (3.0 g, 12 mmoles), Boc-L-phenylalanine (4.0 g, 15 mmoles), Boc-L-alanine (2.2 g, 12 mmoles) and N^{α}-Z,N^{ε}-Boc-L-lysine (8.0 g, 21 mmoles) and the equivalent amounts of dicyclohexylcarbodiimide. The chloride determinations following each deblocking step gave values which were constant within plus or minus 5 %. After the coupling of the final residue, the peptide resin was washed four times with methylene chloride and four times with ethanol, dried, removed from the reaction vessel and weighed. Yield 8.6 g. The increase of 2.1 g corresponds to 68 % of theory, based on the quantity of Boc-L-methionyl resin used.

N^{α}-Z,N^{ε}-Boc-L-lysyl-L-alanyl-L-phenylalanyl-L-isoleucyl-glycyl-L-leucyl-L-methionine amide. Ethanol (80 ml) was saturated with ammonia at 0°C, and left to equilibrate at room temperature and atmospheric pressure, protected from moisture by a drying tube. The solution was added to the peptide resin (8.6 g), and the mixture was stirred for 16 h at room temperature. After filtration and washing three times with ethanol, concentration of the combined filtrates *in vacuo* and crystallization of the syrupy residue from acetic acid/ethanol (by volume 10/90) yielded only a small quantity of crystalline product, which was dried for 2 days. Yield 84 mg, m.p. 247—249°C. (Found: C 59.5; H 7.8; N 11.7; S 3.4. Calc. for $C_{50}H_{77}N_9O_{11}S,C_2H_5OH$ (1058.4): C 59.0; H 7.9; N 11.9; S 3.0). To obtain a more complete cleavage the peptide resin was suspended in ethanol (160 ml) of 0°C, which had been saturated with dry ammonia at 0°C. After stirring by magnet for 96 h at room temperature in a sealed flask, the resin was filtered off and briefly warmed to 70°C with two successive 125 ml portions of ethanol. The resin was finally extracted for 10 min with acetic acid (100 ml) of 40°C. Removal of the solvents under reduced pressure on a rotary evaporator at a bath temperature not exceeding 35°C yielded a crude oil (2.6 g). On crystallization from acetic acid/ethanol as above, the product was

obtained as a faintly yellow crystalline mass, which was dried as above. Yield 1600 mg, m.p. interval 235—243°C. (Amino acid analysis, given in mole per 1058 g of substance: Lys 0.85; Gly 1.09; Ala 0.96; Met 0.74; Ile 1.04; Leu 1.07; Phe 1.05. Conditions of hydrolysis: 24 h at 110°C in 6 N HCl in an evacuated and sealed vial). To see if further cleavage were possible, the remaining resin was stirred for 30 min with sodium-distilled liquid ammonia (30 ml) at atmospheric pressure, protected from moisture by a drying tube. Working up as described above produced 150 mg of white powder. A renewed treatment with liquid ammonia resulted in a negligible yield. Thus the total yield of crude product was 1834 mg, *i.e.* 43 % of theory based on the quantity of Boc-L-methionyl resin used.

In thin-layer chromatography in three solvent systems all three cleavage products showed the same pattern. In solvent systems S2 and S3 they were practically homogeneous, with R_F 0.8 and 0.7, respectively. In S1, however, there were spots with R_F 0, 0.1, 0.3, 0.7, and a faint one with R_F 0.8. The main component had R_F 0.7, and because of the good separation in this system a preparative fractionation of the combined crude products (585 mg) was made on an LKB 4200 (LKB Produkter AB, Stockholm) column (60 × 2.5 cm) filled with silicagel (Kieselgel HF 254, E. Merck AG), using an LKB "Uvicord" to monitor the effluent at 254 mμ (chromatogram Fig. 1). After concentration on

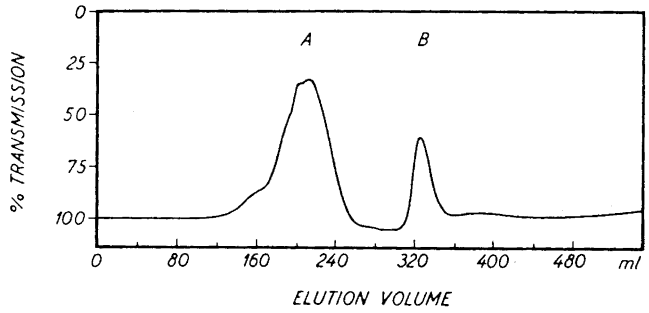

Fig. 1. Purification of the protected heptapeptide amide on silicagel. 585 mg applicated, dissolved in acetic acid (5 ml). Elution with solvent system S1 (flow: 18 ml/h) at room temperature.

a rotary evaporator under reduced pressure at a bath temperature not exceeding 35°C, the solvent containing fraction A was removed by freeze-drying to give a white, voluminous powder (405 mg). In thin-layer chromatography (S1) only small quantities of the

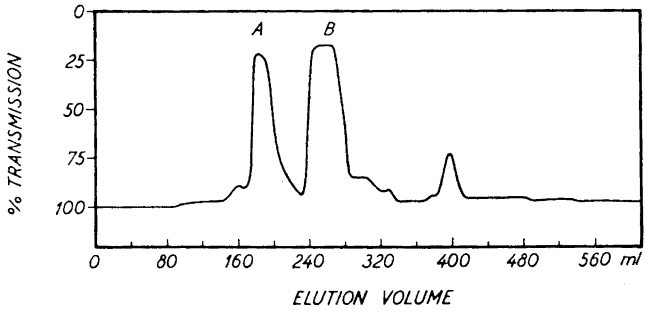

Fig. 2. Purification of the protected heptapeptide amide on Sephadex LH-20. Applicated 301 mg in solvent system S1 (5 ml) and eluted with the same (flow: 10 ml/h) at room temperature.

contaminants with R_F 0.3 and 0.8 remained. On standing, however, especially in solution, the substances with R_F 0.1 and 0.3 were observed to increase in quantity. On application of the freeze-dried product (301 mg) on an SR 25/100 (Pharmacia Fine Chemicals AB, Uppsala) column (100 × 2.5 cm) filled with Sephadex LH-20 (chromatogram Fig. 2) the protected peptide was isolated from fraction A in a chromatographically pure state on freeze-drying; fraction B contained only tolidine-negative impurities. A compact mass of white crystals (120 mg) was deposited on standing for several days in acetic acid/ethanol (by volume 50/50) at room temperature. Renewed application on the column of the freeze-dried intermediary fraction between peaks A and B produced a further 25 mg after crystallization. The yield of crystalline protected heptapeptide amide was thus 145 mg, corresponding to an over-all yield of 14 % of theory, based on the quantity of Boc-L-methionyl resin used; m.p. 248–251°C. (Found: C 59.6; H 7.6; N 11.6; S 3.1. Calc. for $C_{50}H_{77}N_9O_{11}S,C_2H_5OH$ (1058.4): C 59.0; H 7.9; N 11.9; S 3.0. Amino acid analysis, given in mole per 1058 g of substance: Lys 0.87; Gly 0.96; Ala 0.96; Met 0.72; Ile 0.96; Leu 0.97; Phe 0.93. Conditions of hydrolysis as above). $[\alpha]_D^{25} = -34.4°$; $[\alpha]_{578}^{25} = -37.3°$ ($c = 0.4$ in acetic acid). Results of the bioassay are given in Table 2.

Table 2. Bioassays. The activity is compared on a weight base.

	Guinea-pig ileum	Rabbit blood pressure
	(Bradykinin = 1)	
N^{α}-Z,N^{ε}-Boc-heptapeptide amide	0.02 [a]	0.05
N^{α}-Z-heptapeptide amide trifluoroacetate	1.2	1.0

[a] Only partially dissolved.

N^{α}-Z-L-lysyl-L-alanyl-L-phenylalanyl-L-isoleucyl-glycyl-L-leucyl-L-methionine amide trifluoroacetate. A sample of the protected peptide amide (70 mg, 0.066 mmole) was dissolved in anhydrous trifluoroacetic acid (2 ml, 26 mmole) and left to stand for one hour at room temperature. The trifluoroacetate was isolated by precipitation with sodium-distilled ether (20 ml), centrifugation, and decantation of the supernatant. The product was dissolved in acetic acid and freeze-dried to give a white, voluminous powder (70 mg, 84 %); m.p. 220–225°C with sintering from 215°C. (Found: N 10.0; S 2.7. Calc. for $C_{45}H_{69}N_9O_9S,3\ CF_3COOH$ (1254.2): N 10.1; S 2.6). Thin-layer chromatography: $R_{FS2} = 0.5$; $R_{FS3} = 0.3$; $[\alpha]_D^{25} = -27.6°$; $[\alpha]_{578}^{25} = -29.1°$ ($c = 0.5$ in acetic acid). In paper electrophoresis at 3000 V and pH 2.0 in formic acid/acetic acid/water (by volume 2/8/90) for 45 min on Whatman No. 1 paper the trifluoroacetate was practically homogeneous with a mobility of 0.41 (reference compound: N^{α}-dansyl-L-arginine). Results of the bioassay are given in Table 2.

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