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A Synthesis of Thyrotropin-Releasing Factor

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A procedure is given for the synthesis of thyrotropin-releasing factor (TRF), a tripeptide L-pyrogultamyl-L-histidyl-L-proline amide. Benzyloxycarbonyl(Z)-glutaminyl-histidyl-proline amide (III) is obtained as an intermediate by the coupling of Z-glutamine *p*-nitrophenyl ester with histidyl-proline amide which is derived from the crystalline Z-dipeptide amide. Deprotection of III and the subsequent cyclization produce TRF in a moderate yield. Compound III and the corresponding N^{α}-acetyl derivative have little or no TRF activity.

Recently the structure of thyrotropin-releasing factor (TRF, or thyrotropin-releasing hormone, TRH), isolated from porcine[1] and ovine hypothalami,[2] has been elucidated to be a tripeptide L-pyroglutamyl-L-histidyl-L-proline amide. Both a synthetic preparation of the tripeptide amide and natural TRF (porcine) have been shown to be active in man.[3] This important finding as well as the fact that porcine and ovine hormones are chemically identical suggests that TRF is not species-specific among a wide variety of mammals. Because of its very minute occurrence in nature, it seems difficult to isolate TRF from natural sources in quantities sufficient for biochemical investigations and for clinical use. A chemical synthesis of this tripeptide amide will, therefore, become of extreme importance to meet such requirements. Some syntheses have appeared already.[4] We also wish to report a simple procedure for synthesizing the hormone. The present synthesis consists of a step-by-step elongation from C-terminal of the peptide chain, using a benzyloxycarbonyl group for N^{α}-protection, to obtain glutaminyl-histidyl-proline amide which undergoes facile transformation into the corresponding pyroglutamyl peptide (TRF). The procedure is outlined in Fig. 1.[5]

Benzyloxycarbonyl-proline amide, derived from the corresponding acid by a mixed anhydride method, was converted into the crystalline free base of proline amide (I) by catalytic hydrogenolysis. Coupling of I with benzyloxycarbonyl-histidine azide[6] yielded a crystalline dipeptide, benzyloxycarbonyl-histidyl-proline amide (II), in 85 per cent yield. Compound II was treated with hydrogen bromide in acetic acid, followed by coupling with benzyloxycarbonyl-glutamine *p*-nitrophenyl ester[7] to give benzyloxycarbonyl-glutaminyl-histidyl-proline amide (III). A crude preparation of compound III was partially purified by chromatography on a silica gel column with methanol-chloroform (1:3) as solvent. Further purification was performed on a column of carboxymethyl(CM) cellulose using an ammonium acetate buffer with a linear concentration gradient. The effluent solution was monitored at 245 mμ in order to detect the peptide emerging from the column. The pure preparation of III thus obtained was submitted to deprotection with hydrogen bromide in acetic acid. The resulting hydrobromide of glutaminyl tripeptide (IV) was converted into the acetate by passing through a column of an anion-exchange resin (acetate form). The final step leading to the formation of the desired

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5) All amino acid residues are of the L-configuration. The abbreviated designation of amino acids, peptides and their derivatives is in accordance with the proposal of the IUPAC–IUB Commission of Biochemical Nomenclature, which appeared in *Biochemistry*, **5**, 2485 (1966); *ibid.*, **6**, 362 (1967).

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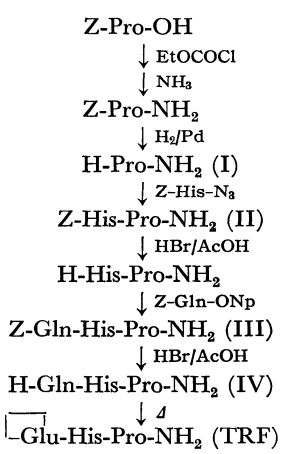

Fig. 1. Synthesis of pyroglutamyl-histidyl-proline amide (TRF).[5]

pyroglutamyl peptide (TRF) is a brief heat treatment of IV (acetate) in acetic acid. The reaction was followed by thin-layer chromatography (silica gel); IV and TRF have R_f values of 0.05 and 0.24, respectively, in methanol-chloroform (1:1) as solvent. Purification of crude TRF was easily achieved by chromatography on a CM cellulose column using an ammonium acetate buffer. The TRF preparation thus obtained was found to be homogeneous to Pauly reagent in thin-layer chromatography (E. Merck Cellulose F, in *n*-butanol:acetic acid:pyridine:water=30:6:20:24, BAPW) and its $[\alpha]_D$ was identical with a literature value[4a] within the error of measurement. The elemental and the amino acid analyses were also in good accordance with the theoretical values.

TABLE 1. THYROTROPIN-RELEASING ACTIVITY OF SYNTHETIC TRF AND RELATED COMPOUNDS[10]

Method	Compound[a] and dose	Number of animals	TRF activity (%increase in blood radioactivity)
A[8]	Saline	4	111.3±24.0
	TSH standard 0.2 mU	6	131.7±14.7
	TSH standard 0.4 mU	6	144.8±17.4
	Synthetic TRF 2 ng	5	153.0± 9.8
	III 2 ng	7	95.0± 5.3
	V 2 ng	7	89.6± 5.4
B[9]	Saline	6	99.5±10.4
	TSH standard 0.4 mU	5	244.0±25.0
	Synthetic TRF 20 ng[b]	7	328.4±27.7

a) TSH=Thyrotropin, III=Z-Gln-His-Pro-NH_2, V=Ac-Gln-His-Pro-NH_2
b) Dose per 100 g body weight

The synthetic TRF was then subjected to biological assays. An in vivo thyrotropin-releasing activity in mice was assayed by the method of Schally *et al.*,[8] and the effect of elevating the blood levels of TSH in rats was estimated by the McKenzie method.[9] Some results are presented in Table 1, in which the data on compound III and acetyl-glutaminyl-histidyl-proline amide (V), derived from IV by acetylation in aqueous pyridine, are also shown.[10] A level as low as 2 ng of the present TRF preparation can release thyrotropin (TSH) in mice, whereas both III and V are devoid of activity at least at the same level. These data as well as those of the chemical characterization are likely to provide evidence for the identity of our synthetic TRF with the natural hormone.

Experimental

All melting points were uncorrected.

Benzyloxycarbonyl-proline Amide. A solution of benzyloxycarbonyl-proline (24.9 g, 0.1 mol) and triethylamine (15.3 m*l*, 0.11 mol) in anhydrous tetrahydrofuran (250 m*l*) was chilled in an ice-salt bath (below −10°C) and to this was added dropwise ethyl chloroformate (10.5 m*l*, 0.11 mol). The mixture was stirred for 10 min, and then concentrated aqueous ammonia (28%, 19.2 m*l*) was introduced. After the mixture had been stirred at room temperature for 30 min, the solvent was removed by evaporation *in vacuo*. To the residue were added ethyl acetate (30 m*l*) and water (30 m*l*), and the mixture was shaken vigorously. The organic phase was further washed with N hydrochloric acid, 5% sodium bicarbonate and water, dried over sodium sulfate and evaporated *in vacuo* to give an oily residue which crystallized from ethyl acetate-petroleum ether; yield 20.1 g (81%), mp 90—91° C, $[\alpha]_D^{27}$ −35.0±0.4° (*c* 2.06, ethanol). Lit,[11] mp 94°C, $[\alpha]_D^{23}$ −33.8° (*c* 2, ethanol).

Found: C, 63.17; H, 6.55; N, 11.53%. Calcd for $C_{13}H_{16}N_2O_5$: C, 62.89; H, 6.50; N, 11.28%.

Benzyloxycarbonyl-histidyl-proline Amide (II). Benzyloxycarbonyl-proline amide (2.2 g, 9 mmol) was hydrogenolyzed over palladium in methanol for 1.5 hr. Removal of the solvent yielded *proline amide (I)* as a sirupy residue which crystallized upon addition of ether; wt. 1.24 g, mp 101—102°C, $[\alpha]_D^{23}$ −86.5±1.2° (*c* 1.08, methanol).

An ethyl acetate solution of benzyloxycarbonyl-histidine azide (derived from 4.1 g (13.5 mmol) of the hydrazide in the usual manner)[6] was added to compound I (1.2 g) obtained above and the mixture was kept at 4°C overnight. Removal of the solvent by evaporation *in vacuo* yielded a sirupy residue which was crystallized from methanol-water. After recrystallization from water the product amounted to 2.95 g (85%); mp 102—104 °C, $[\alpha]_D^{24}$ −40.7±0.7° (*c* 1.13, methanol).

Found: C, 58.56; H, 6.10; N, 17.48%. Calcd for $C_{19}H_{23}N_5O_4 \cdot 1/4H_2O$: C, 58.52; H, 6.07; N, 17.96%.

Benzyloxycarbonyl-glutaminyl-histidyl-proline Amide (III). Compound II (1.54 g, 4 mmol) was dissolved in saturated hydrogen bromide in acetic acid (15 m*l*) and the mixture was allowed to stand at room temperature for 40 min, followed by the addition of ether to yield fine precipitates. The precipitates (1.98 g) were dissolved in dimethylformamide (25 m*l*) and to this was added triethylamine (1.68 m*l*, 12 mmol). The hydrobromide of triethylamine which separated was filtered off and benzyloxycarbonyl-glutamine *p*-nitrophenyl ester (1.61 g, 4 mmol)[7] was added to the filtrate. The

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9) J. M. McKenzie, *ibid.*, **63**, 372 (1958).
10) The assays were performed by Dr. Masahiro Sakoda, Kobe University School of Medicine. We thank Dr. Sakoda for permission to include these data in the present communication.
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mixture was kept at 4°C for 65 hr and evaporated *in vacuo*. The sirupy residue was triturated with ethyl acetate and the mixture was filtered off to collect precipetates which were washed with ethyl acetate and ether and dried *in vacuo* (2.65 g) The precipitates were dissolved in methanol-chloroform (1:3 by vol.) and the solution was submitted to a column of silica gel (E. Merck, 0.05—0.2 mm, 90g) which had been prepared with methanol-chloroform (1:3). The same solvent system was used for elution. Fractions which gave a single component (R_f=0.35, sulfuric acid charring) on thin-layer chromatography (Silica gel G, methanol-chloroform (1:2) as solvent) were pooled and evaporated *in vacuo* to afford a residue which solidified upon treatment with ethyl acetate (1.98 g). The partially purified material (0.80 g) was chromatographed for further purification on a column (1.7×53 cm) of carboxymethyl cellulose (Serva, 0.56 meq/g) using an ammonium acetate buffer (pH 5.0, 2000 m*l*) with a linear concentration gradient of 0.005—0.1 M. Ten-m*l* fractions were collected and their absorptivity at 245 mμ was recorded. Fractions (tubes 41—52) corresponding to a major peak were pooled, evaporated and lyophilized; wt. 0.49 g (53%), $[\alpha]_D^{25}$ −60.4 ±1.1° (*c* 0.88, water), −46.1±0.9° (*c* 0.98, methanol).

Found: C, 52.70; H, 6.00; N, 17.00%. Calcd for $C_{24}H_{31}N_7O_6 \cdot 1/2CH_3COOH \cdot 3/2H_2O$: C, 52.62; H, 6.36; N, 17.18%.

Pyroglutamyl-histidyl-proline Amide (Thyrotropin-releasing Factor, TRF). To compound III (0.20 g) was added saturated hydrogen bromide in acetic acid (*ca.* 5 m*l*) and the mixture was kept at room temperature for 60 min, followed by precipitation by the addition of ether. The resulting precipitates were filtered off, washed with ether and dried *in vacuo* to give *glulaminyl-hislidyl-proline amide hydrobromide (IV)*; wt. 0.26 g, $[\alpha]_D^{25}$ −13.8±0.5° (*c* 0.98, methanol).

Found: C, 30.91; H, 5.03; N, 15.64%. Calcd for $C_{16}H_{25}N_7O_4 \cdot 3/2HBr \cdot 2H_2O$: C, 31.11; H, 5.14; N, 15.87%.

The hydrobromide (IV) obtained above (0.25 g) was dissolved in water and the solution was passed through a column (1.2×10 cm) of Amberlite CG—400 (acetate form); the column was washed with portions of water. The aqueous solutions were combined and evaporated *in vacuo* at a bath temperature of 50°C. The residue was dissolved in glacial acetic acid (20 m*l*) and the solution was heated at 95°C for 15 min. After removal of the solvent by evaporation *in vacuo* the residue was subjected for purification to chromatography on a column (1.7×30 cm) of carboxymethyl cellulose (Serva, 0.56 meq/g) using an ammonium acetate buffer (pH 5.0, 1440 m*l*) with a linear concentration gradient of 0.005—0.1 M. Fractions (7.5 m*l*/tube) were collected and their absorptions at 245 mμ were recorded. There were two peaks, I (tubes 8—10) and II (31—42), on the chromatogram and the tubes corresponding to peak II were pooled and evaporated *in vacuo*. The resulting glassy residue was dried *in vacuo* and then precipitated from acetic acid-ether to yield pure TRF as hygroscopic colorless powder; wt. 0.11 g, $[\alpha]_D^{25}$ −43.4±0.8° (*c* 1.01, 95% acetic acid). Lit,[4a] $[\alpha]_D^{25}$ −44.8° (*c* 1, 95% acetic acid). Homogeneous on thin-layer chromatography (Cellulose F, E. Merck) to Plauy reagent; R_f=0.47 (BAPW). Amino acid ratios in acid hydrolysate:[12] His 1.00, Glu 0.90, Pro 1.14, NH_3 1.01.

Found: C, 50.63; H, 6.56; N, 20.78%. Calcd for $C_{16}H_{22}N_6O_4 \cdot 1/2CH_3COOH \cdot 1/2H_2O$: C, 50.86; H, 6.28; N, 20.94%.

Acetyl-glutaminyl-histidyl-proline Amide. Cmopund IV (0.156 g) was dissolved in 90% pyridine (4 m*l*) at 0°C, and to this solution was added acetic anhydride (0.1 m*l*) and the mixture was kept at 0°C for 90 min. After addition of water (2 *ml*) the mixture was evaporated *in vacuo*. The residue was dissolved in water and the solution was passed through a column (1.2×9 cm) of Amberlite CG-400 (acetate); the column was washed with portions of water. The aqueous solutions were combined and evaporated *in vacuo* to give a residue which was reprecipitated from acetic acid-ether; yield 0.098 g (80%), $[\alpha]_D^{25}$ −50.5±0.8° (*c* 1.11, methanol). Thin-layer chromatography (Cellulose F, F. Merck); R_f=0.45 (BAPW).

Found: C, 46.55; H, 6.40; N, 19.65%. Calcd for $C_{18}H_{27}N_7O_5 \cdot 1/2CH_3COOH \cdot 2H_2O$: C, 46.81; H, 6.82; N, 20.11%.

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