BIBLIOGRAPHIE

[1] *W. Steinkopf*, Liebigs Ann. Chem. *413*, 333 (1916).
[2] *H. D. Hartough*, dans «Thiophene and its derivatives», p. 512, Interscience Publishers, New-York 1952.
[3] *W. Steinkopf*, Liebigs Ann. Chem. *403*, 17 (1914).
[4] *H. Y. Lew & C. R. Noller*, J. Amer. chem. Soc. *72*, 5715 (1950).
[5] *L. M. Jackman & S. Sternhell*, dans «Applications of Nuclear Magnetic Resonance, Spectroscopy in Organic Chemistry», Vol. 5, Pergamon Press, London 1968.
[6] *B. Miche de Malleray*, Helv. *54*, 343 (1971).

38. An Efficient Synthesis of Thyrotropin Releasing Hormone (TRH)

by **Pierre Rivaille** and **Gérard Milhaud**

Laboratoire associé au C.N.R.S., Laboratoire des Isotopes
Service de Biophysique, Faculté de Médecine St-Antoine
27, rue Chaligny, 75 Paris 12e, France

(26. XII. 70)

Summary. A convenient and simple synthesis of L-pyroglutamyl-L-histidyl-L-prolinamide is described. Two pathways using the solid phase technique have led to the pure tripeptide.

Thyrotropin releasing hormone (TRH) was isolated from porcine hypothalamus by *Schally et al.* [1] and from ovine hypothalamus by *Guillemin et al.* [2], and proved to be identical with L-pyroglutamyl-L-histidyl-L-prolinamide [3]. A classical synthesis of this tripeptide was reported recently [4]; the solid phase technique [5] used here gives better yields, is simpler and quicker.

The following reaction scheme was used. *t*-Butyloxycarbonyl (Boc) is introduced in proline, glutamic acid or glutamine as the amine protecting group [6] using Boc-azide at constant alcaline pH. The purity of the Boc-aminoacids was checked by measuring the rotation and by thin-layer chromatography. The histidine imidazole group was protected with *o,p*-dinitrophenyl before condensation, as selective removal of this group can easily be performed by thiolysis at pH 8 [7].

Protected histidine was reacted with proline linked on the chloromethylresin in the presence of dicyclohexyl-carbodiimide (Dcc). The glutamyl residue was introduced in two ways: (A) Boc-pyroglutamic acid was condensed in the presence of Dcc; (B) Boc-glutamine dinitrophenylester [8] was reacted with the dipeptide linked to the resin in dimethylformamide. The tripeptide was split from the resin by aminolysis [9], the imidazole protecting group removed, and the glutamine converted into pyroglutamic acid by boiling acetic acid [10].

The tripeptide was purified by Sephadex G10 filtration followed by silica-gel chromatography. The pure material did not react with ninhydrin, as could be anticipated due to the presence of prolinamide and pyroglutamic acid. After silica-gel thin layer chromatography in several solvant systems [11], the product gave a single spot when detected with *Pauly*'s reagent. The optical rotation was of the order of the one published [4]. After acid hydrolysis, the three initial aminoacids could be detected by spraying the thin layer chromatoplaque with ninhydrin.

Experimental. – 1. *Preparation of Boc-L-proline, Boc-L-glutamic acid and Boc-L-glutamine.* Radiometer autotitrator TTT was used and the reaction time extended to 1,5 time of the usual duration to increase yields.

	pH	Time	Yield	m.p.	$[\alpha]_D$	$c = 1$
Boc-Pro	8.6	2 h	96%	134°	−60,0°	(AcOH)
Boc-Glu	10.0	18 h	97%	116–118°	−16,1°	(MeOH)
Boc-Gln	10.3	24 h	82%	158°	− 3,0°	(EtOH)

Thin layer chromatography with silica gel *Merck* 5721/0025 was performed using as solvants $CHCl_3$-MeOH-AcOH (85/10/5, *v/v*).

2. *N(im)-DNPN-α-Boc-L-histidine.* L-histidine methylester dihydrochlorid was prepared [12] (yield 80%, m.p. 127–130°, $[\alpha]_D = +16,0°$, $c = 1$ (MeOH)) and the amine group protected with Boc in pyridine according to [13] (yield 82%, m.p. 137°, $[\alpha]_D = -14°$, $c = 1$ (MeOH)); finally, dinitrofluorobenzene was reacted with the imidazole group according to [7] (yield 62%, m.p. 94°, $[\alpha]_D = 55,3°$, $c = 1$ (ethyl acetate)).

3. *Boc-L-pyroglutamic acid* was prepared according to [14]. This compound is easier to purify by crystallization than Boc-glutamic acid [15].

4. *Preparation of Boc-L-proline linked to the resin, R.* Bio-beads resin SX2 200–400 mesh was reacted with chloromethyl-methyl-ether [16] in the presence of tin(IV) chloride. The chloride content of the dried resin was 1,37 mMol/g. Boc-proline (6,44 g) was heated at 90° for 50 hours with the chloromethylresin (2,1 g) in presence of dry ethanol (6 ml) and trimethylamine (0,42 ml). The resin content was 0,117 mMol Boc-Pro/g.

5. *Preparation of R-L-prolyl-L-histidyl-N(im)-DNP-α-Boc-pyroglutamic acid (procedure A).* R-Boc-proline (1,37 g) was introduced in the apparatus described by *Merrifield* [5] and shaken in the presence of dioxane (three portions of 10 ml for 5 min each), HCl 4N/dioxane (10 ml, 30 min), dioxane (3 × 10 ml, 5 min each), $CHCl_3$ (3 × 10 ml, 5 min each), triethylamine/$CHCl_3$ (1:9, *v/v*) (10 ml, 10 min), $CHCl_3$ (3 × 10 ml, 5 min each), CH_2Cl_2 (3 × 10 ml, 5 min each). Then N(im)-DNP-N-α-Boc-His (647 mg) was suspended in 6 ml CH_2Cl_2 and shaken for 5 min in presence of the resin; Dcc (500 mg/ml CH_2Cl_2) (0,87 ml) was added and shaken for 150 min, then CH_2Cl_2 (3 × 10 ml, 5 min each). The whole procedure was repeated, then Boc-pyroglutamic acid (481 mg) was introduced. The protecting group of pyroglutamic acid was removed with HCl 4N/Dioxane and the resin washed with acetic acid and ethanol. The weight increase of the dried resin was 56 mg (yield: 80%).

6. *L-histidyl-N(im)-DNP-L-prolinamide-L-pyroglutamyl.* The resin from § 5 was suspended in dry methanol saturated with NH_3 at 0° and shaken for 48 hours at room temperature [9]. After filtration, the resin was washed with methanol and the solvent removed under vacuum at low temperature.

7. *L-pyroglutamyl-L-histidyl-L-prolinamide.* The residue was dissolved in H_2O/CH_3OH (1:1, *v/v*) (10 ml) and the pH adjusted to 8 with sodium carbonate. Thioethanol was added and the mixture left overnight at room temperature [7]. Addition of glacial acetic acid brought the pH to 3,5. The solvents were removed under vacuum; the residue was dissolved in water and dinitrophenol was extracted with ether. The aqueous layer was freeze-dried, taken up in water, and desalted by Sephadex G10 filtration. The head fractions reacting with *Pauly*'s reagent were collected and freeze-dried. The residue was purified by column-chromatography on silica gel (*Merck* 7734, 70–325 mesh; eluent: $CHCl_3$–CH_3OH (30:60, *v/v*)). The fractions giving a color reaction with *Pauly*'s reagent were collected, and the solvents removed under vacuum at room temperature. The residue (50 mg) was dissolved in acetic acid and freeze-dried. The product is hygroscopic. $[\alpha]_D = 40°$ ($c = 1$; AcOH), yield 73% from resin linked proline.

8. *Preparation of R-L-prolyl-L-histidyl-N(im)-DNP-Nα-Boc-L-glutamine (procedure B).* The procedure described in § 5 was applied: to R-L-Boc proline (840 mg) and N(im)-DNP-Nα-Boc-L-histidine (200 mg), but purified dimethylformamide was used to wash the resin instead of CH_2Cl_2; dinitrophenyl-L-Boc-glutamine [8] (m.p. 154-155°, $[\alpha]_D = 35°$, ($c = 1$; dimethylform-

amide)) (382 mg) was allowed to react during 3 hours. The resin was processed as described: the weight increase amounted to a 89% yield.

9. *Cyclization of* L-*glutaminyl*-L-*histidyl*-L-*prolinamide.* The repetition of the steps described in § 6 for aminolysis and § 7 for thiolysis has led to L-glutaminyl-L-histidyl-L-prolinamide. The product was boiled 2 min in glacial acetic acid to convert the glutamyl into the pyroglutamyl group [10]. The mixture was purified as described. The product is hygroscopic. $[\alpha]_D = -39°$, ($c = 1$; AcOH), yield 60% from the resine linked prolin.

10. *Comparison of the peptides prepared by the procedures A and B.* Both products exhibit the same chromatographic behaviour in several solvent systems [11]. After acid hydrolysis (6N HCl, 24 h under vacuum) and thin layer chromatography in 70% ethanol – H_2O (95:5 *v/v*), the two preparations gave rise to the three initial aminoacids.

11. *Biological activity in Man.* At the dose of 200 μg, these preparations are able to elicit a large increase of circulating TSH in 8 normal children as well as in 3 untreated hypothyroid children [17]; they have been used to differentiate hypothalamic from hypophyseal insufficiency [17]. No side effect was noted after intravenous administration.

The technical help of Miss *M.-O. Delaunay* is gratefully aknowledged.

BIBLIOGRAPHY

[1] *A. V. Schally, C. Y. Bowers, T. W. Reddings & J. F. Barrett,* Biochem. biophys. Res. Commun. *25,* 165 (1966); *A. V. Schally, T. W. Reddings, C. Y. Bowers & J. F. Barrett,* J. biol. Chemistry *244,* 4077 (1969).
[2] *R. Guillemin, R. Burgus, E. Sakiz & D. N. Ward,* C. r. hebd. Séances Acad. Sci. D, *262,* 2278 (1966).
[3] *R. Burgus, T. F. Dunn, D. Desiderio & R. Guillemin,* C. r. hebd. Séances Acad. Sci. D, *269,* 1870 (1969).
[4] *D. Gillessen, A. M. Felix, W. Lergier & R. D. Studer,* Helv. *53,* 63 (1970).
[5] *R. B. Merrifield,* J. Amer. chem. Soc. *85,* 2149 (1963); *86,* 304 (1964).
[6] *E. Schnabel,* Liebigs Ann. Chem. *702,* 188 (1967).
[7] *F. Chillemi & R. B. Merrifield.* Biochemistry *8,* 4344 (1969).
[8] *M. Bodansky & V. du Vigneaud,* J. Amer. chem. Soc. *81,* 5688 (1956).
[9] *M. Bodansky & J. T. Sheehan,* Chemistry and Ind. *1964,* 1423.
[10] *F. Folkers, J. K. Chang & B. L. Currie,* Biochem. biophys. Res. Commun. *39,* 110 (1970).
[11] *J. Bøler, F. Enzmann & K. Folkers,* Biochem. biophys. Res. Commun. *37,* 705 (1969).
[12] *N. C. Davis,* J. biol. Chemistry *223,* 935 (1956).
[13] *E. Schröder & H. Gibian,* Liebigs Ann. Chem., *656,* 190 (1962); *K. Hofmann, W. Haas, M. J. Smithers & G. Zanetti,* J. Amer. chem. Soc. *87,* 631 (1965).
[14] *E. Schröder & E. Klieger,* Liebigs Ann. Chem. *673,* 196 (1964).
[15] *J. M. Stewart & J. D. Young,* 'Solid Phase Peptide Synthesis', p. 29, Freeman Edit., London 1969.
[16] *R. B. Merrifield,* Biochemistry *3,* 1385 (1964).
[17] *G. Milhaud, P. Rivaille, M. S. Moukhtar, J. C. Job & E. Binet,* C. r. hebd. Séances Acad. Sci. D, *271,* 1900 (1970).