Editors: A. Pecile & E. E. Müller

GROWTH HORMONE AND OTHER BIOLOGICALLY ACTIVE PEPTIDES

Growth hormone and other biologically active peptides

Proceedings of the International Symposium held in Milan September 17-19, 1979

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1980
Excerpta Medica, Amsterdam - Oxford - Princeton

International Congress Series No. 495
ISBN Excerpta Medica 90 219 0424 1
ISBN Elsevier North-Holland 0 444 90122 1

Library of Congress Cataloging in Publication Data
Main entry under title:

Growth hormone and other biologically active peptides.

(International congress series ; no. 495)
Includes bibliographies and indexes.
1. Somatotropin--Congresses. 2. Peptide hormones--Congresses. I. Pecile, Antonio. II. Müller, E. E. III. Series. [DNLM: 1. Somatotropin--Congresses. 2. Somatomedin--Congresses. 3. Neuroregulators--Congresses. 4. Prolactin--Congresses. W3 EX89 no. 495 1979 / WK515 G883 1979]
QP572.S6G76 599.01'927 80-15288
ISBN 0-444-90122-1 (Elsevier North-Holland)

Publisher:
Excerpta Medica
305 Keizersgracht
1000 BC Amsterdam
P.O. Box 1126

Sole Distributors for the USA and Canada:
Elsevier North-Holland Inc.
52 Vanderbilt Avenue
New York, N.Y. 10017

Printed in The Netherlands by Casparie - Amsterdam

International Symposium on Growth Hormone and other Biologically Active Peptides

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FOREWORD

The International Symposium on Growth Hormone was initiated by Professor E. Trabucchi and first held in September, 1967. It is remarkable that the same symposium has been held continuously every four years in the same location (Milan, Italy) and organized by the distinguished Pharmacology Department of the University of Milan. We are grateful to Chair Professor P. Mantegazza and his colleagues, Professors A. Pecile and E.E. Müller, for organizing the Symposium. I should like to point out that the successes of previous symposia and the present one are largely due to the devotion and interest of Dr. Maria Luisa Pecile. I take this opportunity to thank her on behalf of all the participants of the Symposium.

This volume presents the complete manuscripts of the invited speakers at the fourth International Symposium held in Milan, 17–19 September, 1979. It is divided into six sections: methods of hormonal peptide research, growth hormone, prolactin and human chorionic somatomammotropin, somatomedin, hypophysial peptides and peptidergic neurotransmitters, and clinical aspects. There were also 70 short communications but their texts are not included in this volume. It is hoped that this book will be of value to those who are directly concerned with experimental and clinical investigations on growth hormones and related active peptides.

Choh Hao Li

San Francisco, California
April, 1980

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PEPTIDE HORMONE SYNTHESIS: PRESENT STATE OF SOLUTION AND SOLID PHASE METHODS

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The first synthesis of a peptide hormone was that of oxytocin by Vincent du Vigneaud and collaborators in 1953 (11). This synthesis, recognized by the Nobel Prize Award in 1955, also marks the beginning of modern peptide chemistry. It certainly influenced peptide chemists for many years to be predominantly concerned with the synthesis of hormones rather than other important classes of peptides, such as antibiotics. It is interesting to briefly reflect on what was known about naturally occurring peptides in 1953, since this topic was thoroughly reviewed by Bricas and Fromageot (7) in the Advances in Protein Chemistry. Although the existence of many bioactive peptides, including insulin, glucagon, secretin, etc., was well recognized, the chemical structures of only six were available, i.e. of the dipeptides anserine, carnosine and lycomarasmine, the tripeptide glutathione and the folic acid and ergot alkaloid groups. Bricas and Fromageot concluded: "...the number of known naturally occurring peptides is not very great...and very little is known about these substances as a group."

Clearly, the progress that has been made in the past quarter of a century is monumental. Peptide hormones of known structure range in size from the tripeptides thyroliberin (TRH)* and melanostatin (MIF) with molecular weights of 362 and 284 daltons, respectively, to the group of glycoprotein hormones comprising lutropin, follitropin, thyrotropin and choriogonadotropin, with molecular weights of 28-36,000 daltons and up to 240 amino acid residues (review, ref. 32). Their number continues to increase as new peptide hormones are isolated and characterized such as pancreatic polypeptide (25), enkephalins (19) and endorphins (review, refs. 16, 26), and thymus-derived immunostimulatory factors (review, ref. 30).

While several peptide and protein hormones are available to various degrees by isolation from natural sources (e.g. insulin, growth hormone, corticotropin, placental hormones) many others of low natural abundance require chemical synthesis to become accessible in useful amounts

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* Abbreviations follow most IUPAC-IUB recommendations.

for research or pharmaceutical use. An impressive number of peptide hormones and analogues have been synthesized, and rapid methodologic progress has been made both in conventional synthesis in solution and in solid phase procedures (review, refs. 12, 13, 15). Indeed, to some investigators peptide synthesis nowadays appears to be mere routine. However, Table 1 clearly shows that a list of successful syntheses can be readily matched by examples of incomplete work and targets whose synthesis has not yet been attempted. Therefore, far from being mere routine, peptide synthesis remains to be an *"Undiminished Challenge"* (6). We need new methods, but we also need to apply strategies that utilize the efficacy of the available methods more fully. In the words of Theodor Wieland (48), "every synthesis of a complicated polypeptide represents the sum of prolonged intellectual and experimental exertions." Why is this? It is the individuality of each amino acid sequence, the determinant of its unique biological specificity which subjects its "manipulation in synthesis to such diversion, suspense and drudgery as found with no other substances" (48).

Moreover, the demands have greatly increased. Typically, multigram amounts of larger peptides have to be prepared in shorter periods of time and in higher states

TABLE 1. Scope and Constraints in Peptide Hormone Synthesis[a]

Successful Syntheses[b]		Incomplete Syntheses[c]	
Peptide or Group	Size[d]	Peptide	Size[d]
Releasing Factors	3-14	Relaxin	26+21
Enkephalins	5	Vasointestinal Polypeptide, VIP	28
Angiotensins	7-14	Cholecystokinin	33
Kinins	8-17	Big Gastrin	34
Neurohypophyseal Hormones	9	Thymopoietin	49
Substance P, Neurotensin	11-13	Urogastron	53
Melanotropins	13-28	Proinsulin	80-86
Endorphins	15-31	Parathyrin, PTH	84
Gastrin	17	Growth Hormone, GH	191
Motilin	22		
Secretin	27	Synthesis not yet Attempted	
Thymosin α_1	28		
Glucagon	29	Epidermal Growth Factor, EGF	53
Insulin	30+21	Nerve Growth Factor, NGF	118
Calcitonin	32	Somatomedin	
Corticotropin, ACTH	39	Choriomammotropin, CS	191
Gastric Inhibitory Peptide, GIP	43	Prolactin, PRL	199
β-Lipotropin, β-LPH	91	Thyrotropin, hTSH	201
		Lutropin, hLH	206
		Follitropin, hFSH	206-209
		Choriogonadotropin	237-240

[a] A selective rather than a complete listing. [b] Synthetic product is chemically and biologically indistinguishable from the natural hormone. [c] Synthetic product is not homogeneous, not fully active, or otherwise not completely identical with the native hormone. [d] Amino acid residues.

of purity. For example, enkephalins and endorphins are required for clinical studies in 10-50 gram amounts since only clinical tests will permit evaluation of the therapeutic potential of the opioid peptides in mental disorders in man.

Peptide synthesis may be carried out by conventional chemistry in solution or by the Merrifield solid phase method. In either approach, the limitations of our present methodology restrict the selection of target molecules for *routine synthesis* to peptides with 25-30 amino acid residues. This size limitation is quite obvious from Table 1 which shows few successful syntheses of hormones with over 30 residues (calcitonin, ACTH, GIP, insulin) and one, β-lipotropin, with over 50 residues. Syntheses of peptides of more than 50 residues are major pioneering research ventures and any routine chemical synthesis of proteins appears to be a rather distant goal. However, its principle feasibility has been demonstrated in 1969 by the remarkable achievements of Hirschmann and collaborators (18), in approaching a ribonuclease A synthesis by conventional methods in solution, and of Gutte and Merrifield (17) by using solid phase synthesis.

CONVENTIONAL PEPTIDE SYNTHESIS IN SOLUTION

The main problem in the synthesis of larger peptides and proteins by conventional synthesis in solution is the general failure of coupling large intermediate segments in more than trace amounts. This is best illustrated in the unfinished synthesis of ribonuclease T_1 by Hofmann and collaborators (39) in which unsurmountable problems were encountered in attempts to condense the NH_2-terminal 47-peptide with the COOH-terminal 57-peptide. The failure of such large segments to be condensed efficiently is due to their drastically decreased molarity in the reaction mixture, (a) because of their high molecular weight, and (b) because of their poor solubility in suitable organic solvents which generally decreases rapidly with increasing size. It would be of little help, if any, to develop new reagents for more powerful carboxyl activation because the available methods are already very powerful. Indeed, stronger carboxyl activation would be counterproductive by greatly increasing the danger of racemization of the activated terminal amino acid in the carboxyl component. What is needed is a sort of molecular clamp (Fig. 1) to pull the reactive groups of the protected carboxyl (A) and amine components (B) into close proximity and convert the actual peptide bond formation into a *concentration-independent* intramolecular rearrangement. The "Four Component Condensation" of I. Ugi (44) appears to be suitable for this purpose (45) as well as the "Amine Capture" method developed by Kemp *et al.* (22), Fig. 1. While these concepts proved to be feasible for small peptides they have yet to be tested in coupling of large segments.

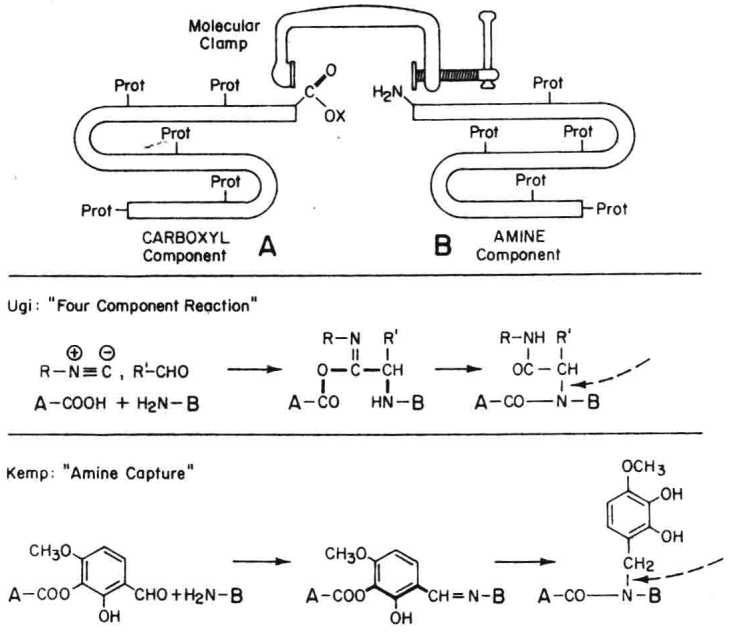


Figure 1. Novel approaches to peptide condensation. The Ugi reaction (44) and amine capture method (22) produce clamp-type intermediates that bring the activated carboxyl group and the amine group of protected (Prot) peptide fragments A and B into close proximity. The desired peptide bonds are then formed by intramolecular rearrangement.

Undiminished challenges in syntheses of large peptides are also faced by the increased danger of racemization, mentioned before. None of the methods used for peptide bond formation is safe from racemization which occurs via intermediate 5(4H)-oxazolone formation (review, ref. 20). Even the azide method, which was long considered to be "racemization-free", gave rise to 40% racemization in a human calcitonin segment condensation (41). The problem is made all the more vexing by the absence of any test to detect the occurrence of racemization directly during synthesis. Such a test would be of immense value.

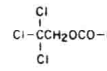
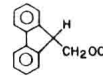
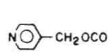
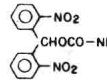
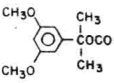
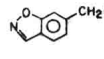
An important challenge continues to be purification to obtain synthetic peptides in homogeneous form. High performance liqid chromatography (HPLC) has been adapted to purification of protected peptides on a multigram scale using prepacked silica gel columns. Free peptides can be purified by a variety of reversed phase HPLC systems (43). β-Lipotropin and human growth hormone have been purified in 75 mg loads. Extension to still larger scale is a challenging task.

STRATEGIES OF FUNCTIONAL GROUP PROTECTION

The proper choice of protecting groups assumes critical importance in the synthesis of larger peptides. Incomplete cleavage at the end of synthesis has been responsible for the reported failure of several syntheses, including lysozyme (23) and Kazal trypsin inhibitor (37). One of the reasons lies, for example, in the current overdependence on graded acidolytic protecting group cleavage. Characteristic for these strategies is the use of the strongest cleaving agents, such as liquid hydrogen fluoride or sodium in liquid ammonia for the treatment of the final sensitive and valuable product. Experience now shows that liquid HF can be a very destructive reagent for peptides of increased chain length (43). Alternative approaches in protecting group strategy are therefore urgently needed.

It appears to be essential that the mildest available deprotecting agent should be used for the final deblocking of the target molecule and that the cleavage be quantitative. This requires the application of chemical selectivity, also referred to as orthogonal strategy (3). A variety of groups have been proposed, for example the benzisoxazolyl-5-methyleneoxycarbonyl (Bic) group by Kemp (21), see Table 2, or the β-(trimethylsilyl)-ethyloxycarbonyl (Teoc) group by Carpino (9).

Table 2. Proposed Protecting Group Combinations Providing Chemical Selectivity and Mild Deblocking of Completed Synthetic Peptides

STRUCTURE	REFERENCE ABBREVIATION	STABILITY	CLEAVAGE	COMBINATIONS N^{α}	COMBINATIONS SIDE CHAIN
	Trichloroethyloxycarbonyl Tcoc; OTce Woodward, 1966	Acid (HF) Base	REDUCTIVE Zn–MeOH, AcOH DMF	Boc Fmoc Bic	Tcoc, OTce
	9–Fluorenylmethyloxycarbonyl Fmoc Carpino & Han, 1970	Acid H_2 – Pd	NONHYDROLYTIC BASE: piperidine, liq. NH_3	Z Boc Fmoc	Fmoc Boc, OtBu, tBu
	Isonicotinyloxycarbonyl Inoc; OPic Veber et al., 1972 Comble et al., 1969	Acid (HF) Base	REDUCTIVE Zn – 50% aq HOAc H_2 – Pd	Boc Z Inoc	Inoc, OPic, Pic (Tyr)
	2,2'–Dinitrodiphenylmethyloxycarbonyl Dnpc; ODnp Patchornik et al., 1970	Acid	PHOTOLYTIC H_2 – Pd HBr – AcOH	Boc Bic Fmoc	Dnpc, ODnp
	2–(3,5–Dimethyloxyphenyl)–propyl(2)–oxycarbonyl Ddz Birr, 1972, 1973	Base	PHOTOLYTIC Mild Acid	Z Boc Picoc	Tcoc, Fmoc Inoc Boc, OtBu, tBu
	Benzisoxazolyl–5–methylene–oxycarbonyl Bic Kemp & Hoyng, 1975	Mild Acid (TFA)	SOLVOLYTIC a) Aprotic Base b) H_2O, pH7 H_2 – Pd	Boc Bic	Bic, OPic Boc, OtBu, tBu

SOLID PHASE PEPTIDE SYNTHESIS

The solid phase method of Merrifield (35) is an ingenious procedural simplification of stepwise peptide synthesis. In the 15 years since its inception it has provided unprecedented stimuli to peptide synthesis. Its most remarkable single achievements include syntheses of a ribonuclease A preparation exhibiting 78% of the activity of the native enzyme by Gutte and Merrifield (17) and of homogeneous 91-peptide ovine β-lipotropin by Yamashiro and Li (51). However, the most profitable and advantageous use of solid phase synthesis may be in rapid synthesis of analogues of a given hormone under optimized conditions, e.g. of gonadoliberin (GnRH), angiotensin, oxytocin and vasopressin, where it has contributed in a major way to the establishment of important structure activity relationships.

The main attractive features of the method are: (a) high speed, permitting up to six amino acid residues to be coupled per day, (b) no insolubility problems of the kind that hamper solution synthesis, (c) ease and convenience of operation, and (d) mechanization and automation.

These advantages, especially the absence of the insolubility problem should enable rapid synthesis of large peptides and proteins. However, the principle shortcomings prevent just that: (a) repeated incomplete (<99%) reactions create product microheterogeneity, and (b) protective group cleavage by differential acidolysis requires final liquid hydrogen fluoride (HF) cleavage of the product from the resin which has caused serious product decomposition in many cases. These shortcomings have been severe for several years resulting in failures of attempted syntheses, e.g. of lysozyme, acyl carrier protein, antibody domains, and pancreatic trypsin inhibitor.

However, a series of significant improvements have been made recently, for example: suppression of loss of peptide from the resin support, by modified supports, e.g., phenylacetamidomethyl or polydimethylacrylamide derivatives; more rapid and efficient coupling by the use of symmetrical anhydrides of Boc-amino acids and trifluoroethanol as an additive; development of various halogenated benzyl-type side chain protective groups of increased acid stability; improved monitoring, e.g. by the picrate method; and proper selection of HF cleavage conditions. As shown in Fig. 2, these modifications, indicated by horizontal arrows in the right-hand margin, have involved almost all operative stages of solid phase synthesis (review, refs. 4, 12, 36). It must be emphasized that the use of these improved procedures is absolutely necessary for obtaining acceptable results. The standard procedures of 1969 (35) have become obsolete and results of synthetic work done nowadays in the old-fashioned manner will be highly questionable.

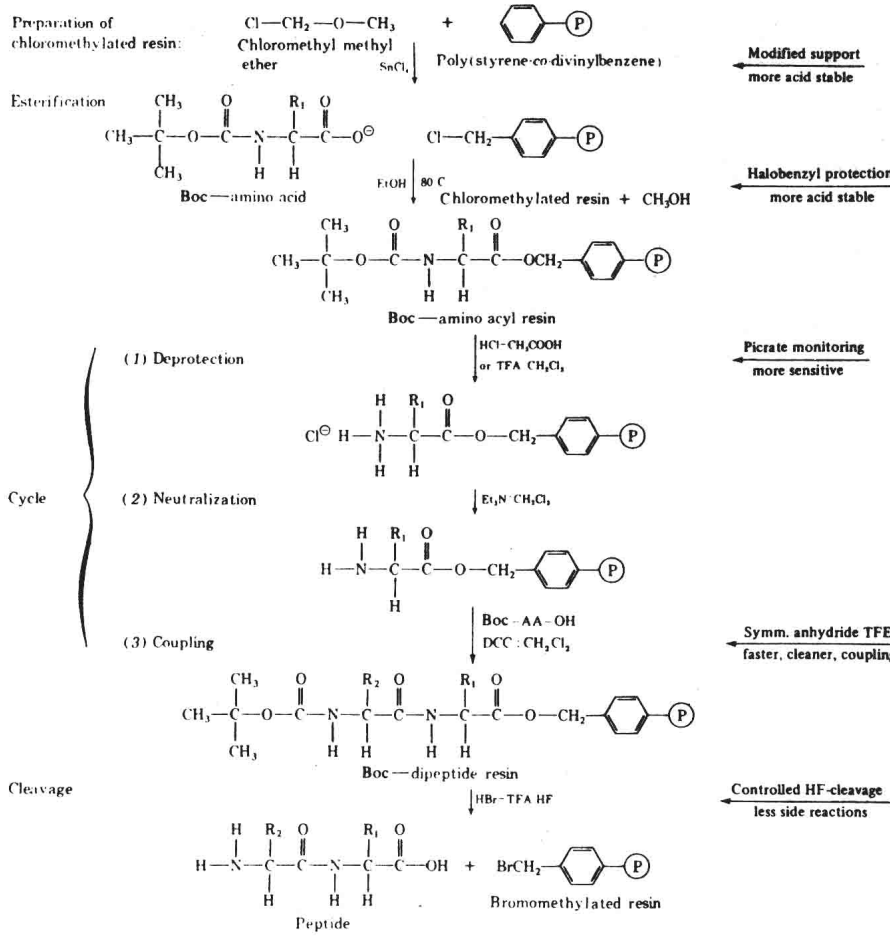


Figure 2. Scheme of solid phase peptide synthesis, showing types of procedural improvements at the right-hand margin. Ⓟ-, solid support; TFA, trifluoroacetic acid; TFE, trifluoroethanol; Et_3N, triethylamine; DCC, dicyclohexylcarbodiimide; Boc-AA-OH, N^{α}-*tert*-butyloxycarbonyl amino acid.

Extensive purification and scrupulous characterization of the final product still remains to be a vital part of solid phase synthesis. The most advanced and powerful separation techniques must always be used. Excellent purification may be achieved by partition chromatography (50), by countercurrent distribution, or by affinity purification. An outstanding example is the synthesis of β-lipotropin by Yamashiro and Li (51) who purified the synthetic material by gel filtration, chromatography on carboxymethylcellulose, and partition chromatography on agarose. The synthetic product was indistinguishable from the natural hormone in its Rf value on partition