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ENZYMATIC PEPTIDE SYNTHESIS

Willi Kullmann

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PREFACE

Nature has provided an abundant collection of enzymes* which are characterized by their comprehensive, functional diversity. In addition to their "natural" roles this multitude of biocatalysts constitutes an enormous and as yet untapped treasure trove for synthetic organic chemists. Although enzymes had been technically exploited long before their catalytic and chemical essence was fully understood, their use as novel catalysts in organic fine syntheses has been the subject of substantial research only during the last 10 to 15 years. Since there exists an enzyme-catalyzed equivalent for most organic syntheses,[3] one can expect that in the near future a steadily growing number of synthetic applications will emerge from this challenging area of enzyme technology.

One may ask, why do enzymes enjoy a wide and still growing popularity as catalysts for organic syntheses? Certainly, enzymes achieve impressive rate enhancements for the chemical processes they promote. However, the most compelling attraction is based on the unique specificity that they display. From the organic chemist's point of view, therefore, the most notable advantage of enzymes is their capacity to combine, in a concerted fashion, different specificities such as stereo- and regiospecificity thereby opening up new possibilities for degrees of control presently unattainable in any other way. Consequently, the efficient production of chiral synthons with the targeted asymmetric centers and functional groups may be best achieved enzymatically. Another point in favor of enzyme-catalyzed syntheses is that the chemical transformation so mediated takes place under relatively mild conditions, i.e., in a largely aqueous environment and in the absence of potentially toxic solvents, at moderate pH values and temperatures normally ranging from 3 to 10 and 20 to 40°C, respectively, under standard pressure. In summary, the rate accelerations, the incorporation of geometric control into normally random chemical processes, and the mild reaction conditions brought about by enzyme-catalysis significantly aid the preparation of products with a high degree of purity. Simultaneously, the safety factor is increased both for the experimenter and for the environment as a whole.

When searching for enzymes with useful catalytic capabilities for peptide synthetic chemistry, the ability of enzymes to function in vitro in the same fashion as they do in vivo comes into question. Unfortunately, the idea of using enzymes which normally mediate in vivo peptide bond formation in preparative scale peptide synthesis is barely feasible. Apart from other drawbacks they are not commercially available and they are exceedingly difficult to prepare. However, an alternative exists in the proteases which for decades were actually considered to be the true in vivo catalysts of protein biosynthesis.[4] The proteases largely fulfill an essential requirement for routine use in peptide synthetic chemistry in that, for the most part, they can be easily isolated from their natural sources or purchased at reasonable prices. In vivo, proteases serve a variety of different functions.[5] Not only are they the catalysts of generalized protein digestion, they also regulate many biological processes. The feature underlying these physiological activities is their proteolytic capacity which enables the proteases either to decompose a given protein altogether or — by selectively cleaving specific bonds — to stimulate the release of biologically active peptides and proteins from their inactive precursors. To date it has been principally the "destructive" property of the proteases which has been exploited in enyzme technology, in the main, for industrial applicaitons. Thus, proteases were used as early as 1907 — in the leather industry — and over the years a large number of additional uses have been developed. In the meantime, the production of proteases has reached some 500 tons/year,[6] i.e., in terms of quantity, proteases are ahead of all other biocatalysts used in enzyme technology.

* The terms enzyme from the Greek ἐν ζύμη (en zyme) for "in yeast" and catalyst from the Greek κατά-λύσις (kata-lysis) for decomposition were coined, respectively, by W. Kühne in 1877 and by J. J. Berzelius in 1835.[1] Moreover, Greek and Arabian alchemists had already gone in search for a catalyst-like substance which they called respectively ξηρίον (xērion, Engl. desiccative powder) and الكسير (al-iksir, Engl. miraculous mixture).[2] This elusive substance — the much-heralded "philosopher" stone' — was thought to mediate the transformation of base metals to precious metals without itself undergoing change.

Given that under physiological conditons, the equilibrium position in a protease-catalyzed reaction largely favors proteolysis, i.e., the cleavage of peptide bonds, it is not surprising that the proteosynthetic potential of the proteases has been largely neglected. However, according to the principle of microscopic reversibility,[7] the proteases do indeed possess the ability to catalyze the synthesis as well as the hydrolysis of a peptide bond. Consequently, it is the equilbrium point of the reaction, not the nature of the enzyme, that decides whether bonds will be made or broken. Thus, if the equilibrium position of a reaction can be shifted away from proteolysis, then the synthesis of peptide bonds, which is negligible under physiological conditions, may proceed to a significant extent. This is by no means a merely theoretical concept as has been demonstrated by a number of successful protease-mediated peptide syntheses (for reviews see References 8 to 12). Indeed with their special properties of stereo- and regiospecificity and the consequent lack of by-products, the use of protease-controlled reactions may well gain preference over the equivalent chemical syntheses in future artificial peptide and protein preparations.

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Willi Kullmann, Ph.D., graduated from the University of Cologne in 1975 with a Diplom degree and received the degree Dr. rer. nat. in Biology in 1977 from the same institution. His doctoral thesis dealt with the chemical synthesis of insulin both via classical methods and the Merrifield solid-phase methods.

In 1978, Dr. Kullmann joined the Max Planck Institute for Biophysical Chemistry in Göttingen (FRG) where his basic interest concerned enzymatic peptide synthesis. In the years to follow, he succeeded in preparing a series of neuropeptides by using the enzymatic approach to peptide synthetic chemistry. Currently, Dr. Kullmann is a Senior Research Associate at the Institute for Cell Biochemistry and Neurobiological Clinic at the University of Hamburg (FRG).

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Chapter 1

INTRODUCTION

The beginnings of peptide synthetic chemistry* can be traced to 1901 when E. Fischer and E. Fourneau reported on the first systematic synthesis of a dipeptide,[1] and further, back to 1882 when T. Curtius unintentionally succeeded in the first in vitro formation of a peptide bond.[2] However, the recent advances in peptide synthetic methodology, cumulating in the fully automatic production of polypeptides, commenced only 30 years ago. The starting point of modern peptide synthetic chemistry was marked in 1953 by the chemical synthesis of the nonapeptide hormone oxytocin by du Vigneaud and his collaborators.[3] Subsequently, further rapid progress has been stimulated principally by the discovery of an ever-increasing number of biologically active peptides. Whereas a review article published in 1953 and entitled *Naturally Occurring Peptides*[4] presented the confirmed structures of only six peptides, the compilation of all currently known peptide structures would grow into a herculean task.

The functional versatility of the peptides is astonishing. Their diverse nature encompasses sweeteners and toxins, antibiotics and ionophores, and chemotactic as well as growth factors. Peptides can act both as stimulators and inhibitors of hormone release. They are involved as morphinomimetics in the pain pathways, and in serving as neurotransmitters, they mediate synaptic communication. They consitute enzyme inhibitors, and conversely, they can function as hormonal messengers to activate appropriate target systems.

The isolation and characterization of a hitherto unrecognized peptide almost invariably entails renewed peptide synthetic activities that may aim at a variety of goals. The classical objective of peptide synthesis is the verification or falsification of primary structures elucidated by sequence analyses of bioactive peptides. Furthermore, synthetic peptides, structurally related to their native counterparts, are effective tools to probe the relationship between peptide structure and biological activity. From a pharmacological point of view it is of particular interest that synthetic analogs of peptide hormones may exhibit unique properties such as superpotency, altered biological specificity, and long-lasting activity. Thus, synthetic peptides may be required for therapeutic purposes, particularly if their natural pendants are not easily obtainable in sufficient quantities.

A novel application of peptide synthetic chemistry is in the production of peptides to be used as immunogens in the generation of antisera specific for proteins of which the peptide represents only a part. Although still in its infancy, this rapidly developing technique not only offers exciting possibilities for raising antigenic determinant specific antibodies but it should also deepen our understanding of the basis of antigenicity. Beyond this lies the creation of entirely artificial peptides with unprecedented primary structures. These truly *ex arte* peptides may be devised either to exhibit putatively nonbiological properties, or to mimic or even enhance commonly known biological activities of their *ex natura* counterparts. Last but not least, peptide synthetic studies may be performed "simply" for the sake of methodological progress.

At present the most frequently used methods of peptide synthesis are those of a chemical nature. The chemical formation of a peptide bond can, in principle, be reduced to four steps (for more detailed descriptions see References 5 to 10; Figure 1).

* The term "peptide" as coined by E. Fischer generally denotes unbranched chain-like molecules consisting of up to 100 amino acid residues, whereas molecules possessing more than 100 residues are commonly referred to as proteins. Peptides may be further classified into oligo- and polypeptides; the former containing between 2 and 10 and the latter 11 to 100 amino acid units.

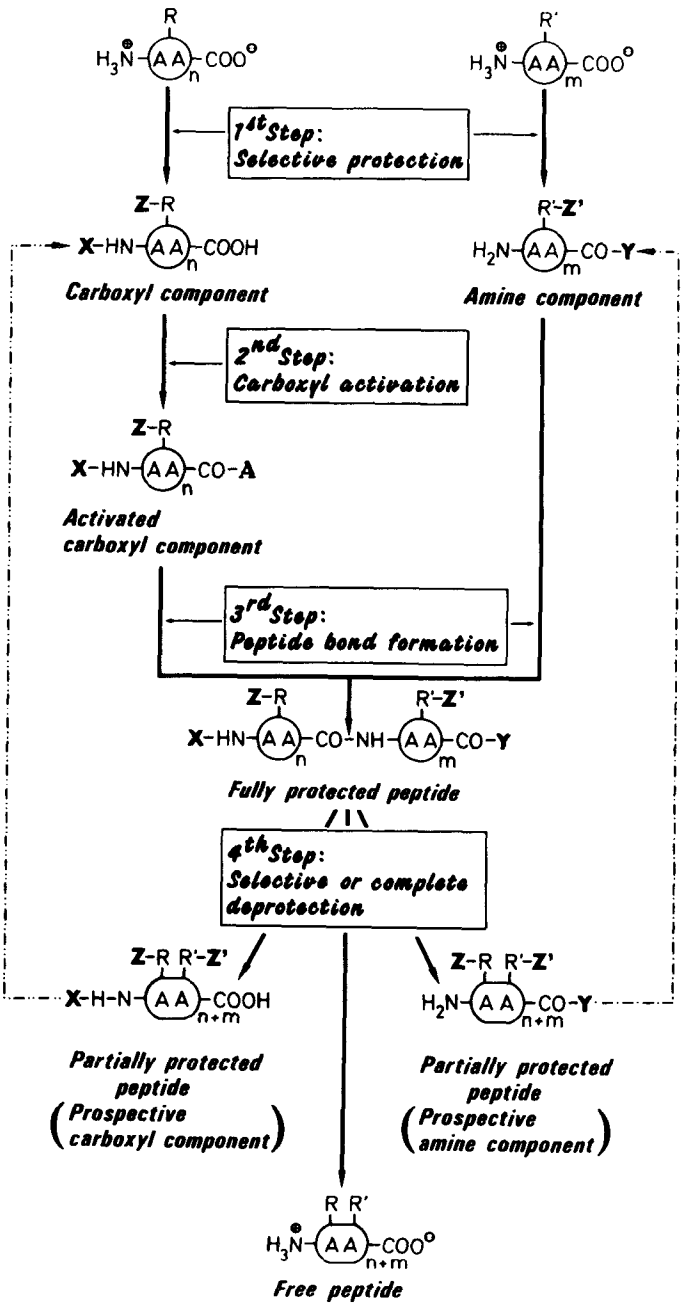

FIGURE 1. Basic scheme of chemical peptide synthesis. AA, amino acid; R,R′, side-chain functionalities; X, α-amine protecting groups; Y, α-carboxyl protecting groups; Z,Z′, side-chain protecting groups; A, activating substituent.

1. The main-chain and side-chain functionalities of the educts — amino acids or peptides — which are not to participate in the reaction must be selectively protected.
2. The carboxyl component must be activated. (The carboxyl component and the amine component contribute the carbonyl group and the imino group, respectively, to the prospective peptide bond.)

3. The peptide bond is formed by coupling the carboxyl component and the amine component via an α-amide linkage.
4. The protecting groups must be removed *in toto*, if the synthesis is completed, or by selective cleavage of the α-amine or α-carboxyl protection, if the synthesis is to be continued.

Originally all synthetic peptides were routinely prepared by conventional solution methods, i.e., the reactants were freely soluble in the reaction media. However, due to B. Merrifield's ingenious innovation of covalently binding the growing peptide chain to an insoluble polymeric support[11] the solid-phase methodology has become increasingly popular over the past 20 years. The most attractive improvement brought about by this procedural simplification is the time-saving and convenient mode of operation and consequently, the prospect of fully automated synthesis.

Numerous peptides have been routinely prepared by solution and solid-phase methods as well as by other techniques such as liquid-phase[12] or alternating solid-liquid phase[13] procedures and remarkable advances have been made in the art of chemical peptide synthesis. Nevertheless, the considerable shortcomings of these methods still impose an "undiminished challenge"[14] upon peptide synthetic chemistry. These limitations arise mainly from the fact that the individual steps of the synthetic pathway are relatively unspecific in nature. Consequently the success of many a synthesis is jeopardized by the appearance of undesired by-products.

To circumvent these problems, an increasing number of organic syntheses is carried out in the presence of enzymes. Due to their stringent specificity, these biocatalysts do not normally permit significant levels of side reactions.

Peptidyltransferase, the enzyme which is responsible for peptide bond formation in vivo, might seem the obvious choice to mediate the enzymatic synthesis of peptides. However, this enzyme is an integral part of the ribosome[15] and its activity is dependent upon the presence of additional ribosomal proteins.[16] Consequently, it is difficult — if not impossible — to isolate this enzyme in a biologically active form.

In contrast, the proteases, which had previously been considered to be the catalysts of protein biosynthesis,[17] are more easily obtainable from their natural sources. Superficially, it may appear incongruous to attempt the synthesis of peptides using proteolytic enzymes. The equilibrium position in a protease-catalyzed reaction is usually far over in the direction of hydrolysis and as a consequence, the reversal of this reaction, i.e., the formation of peptide bonds, represents merely a negligible quantity under physiological conditions. However, the proteases per se cannot be held responsible for this state of affairs. Like other enzymes, they simply accelerate the attainment of equilibrium in a chemical process, whereas the equilibrium point itself, and thus the net synthesis or hydrolysis of the peptide bond, is determined exclusively by thermodynamic factors. Indeed, the proteases do possess the inherent capacity to catalyze both the synthesis and the hydrolysis of a peptide bond, and it is therefore the equilibrium position that actually decides upon the making or breaking of peptide bonds. Consequently, if suitable expedients could be found to shift the equilibrium point of a protease-controlled reaction in favor of the "reverse reaction", the synthesis of peptide bonds may likely become a considerable quantity.

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Chapter 2

REVERSIBLE ZYMO-HYDROLYSIS
A CHRONOLOGY OF ENZYMATIC PEPTIDE SYNTHESIS

The concept of peptide synthesis by reversal of mass action in protease-catalyzed reactions dates back to 1898 when J. H. van't Hoff suggested that the protease "trypsin"* might posses the inherent capacity to catalyze the synthesis of proteins from degradation products originally generated by its own proteolytic action.[2] The rationale behind this idea followed from the applicability of the law of mass action to enzyme-controlled reactions; their reversibility ensuing from the presumed catalytic nature of the process.

The possibility of the participation of hydrolases in not only the degradation but also the assembly of biological macromolecules was first suggested by the phenomenon of the so-called "reversible zymo-hydrolysis", a term introduced in 1898 by A. C. Hill to designate the maltase-catalyzed synthesis of maltose from glucose.[3] Indeed, the earliest reports on glycosidase-, lipase-, and protease-mediated syntheses of glycosides, fats, and peptides were published as early as 1899[3] and 1901,[4,5] respectively, and a further series of studies dealing with enzymatic syntheses of these biomolecules were performed during the following decades (for reviews see References 6 and 7). In contrast, as our present picture of nucleic acid chemistry has evolved only since the early 1950s, it comes as no surprise that the first report of a nuclease-catalyzed formation of oligonucleotides did not appear until 1955.[8]

As mentioned by J. T. Edsall[9] many biochemists, perhaps under the influence of W. Ostwald's "imperative of energetics" (do not waste any energy, but do exploit it),[10] believed that a biochemical process which required free energy to take place could be accomplished with the greatest efficiency by living organisms. As a consequence it was generally taken as granted that the anabolic pathways leading to the synthesis of biological macromolecules were merely reversals of catabolic pathways. This view implicitly suggested that proteins could be prepared by proteolysis in reverse; that is to say, via protease-catalyzed proteosynthesis. The idea of protein biosynthesis by "reversible enzymic hydrolysis" had for some time been considered to be confirmed by the phenomenon of the so-called "plastein-reaction". The term "plastein" was coined in 1901 by Savjalov to designate the precipitate resulting from the addition of rennin to a partial-hydrolysate of fibrin (peptone).[5] Savjalov, while reproducing an experiment performed by his teacher Danilewski in 1886,[11] correctly identified the plastein formation as the outcome of a "proteo-synthetic" process, namely the reverse of the already known "proteo-lytic" action of proteases. Numerous studies on the subject of plasteins were published in subsequent years, particularly during the first decades of the present century (for further details consult References 6 and 7).

For example, plastein formation was observed upon addition of pepsin, papain, trypsin, and chymotrypsin to concentrated solutions of "peptic" partial-hydrolysates. Although the indications were that the plastein represented a complex mixture of small peptides, their chemical nature remained obscure because the complexity of the digestion mixtures prevented detailed characterization of the plasteins with the methods available at that time. It was not until the 1960s that the mechanism of plastein formation was elucidated by using unambi-

* The proteases pepsin and trypsin had already been isolated, respectively, by T. Schwann in 1836 and by W. Kühne in 1877.[1] The designations "pepsin" and "trypsin" were derived from the Greek πεψις (pepsis) for digestion and from τρῦχω (trychō) for to wear out, i.e., to digest. In contrast, the denomination "peptide" has its roots in the term "peptone" (a mixture of small peptides generated from proteins via pepsin-catalyzed digestion) the first four letters of which were combined with the last three letters of "polysaccharide", a term derived from the carbohydrate nomenclature.

gously characterized plastein-forming oligopeptides as protease-specific substrates (*vide infra*, Chapter 9).[12]

In 1938, Bergmann and his collaborators were the first to describe the enzymatic synthesis of well-defined peptides. Thus Bergmann and Fraenkel-Conrat succeeded in preparing via papain-catalysis the dipeptide Bz-Leu-Leu-NHPh from benzoylleucine and leucine anilide,[13] and Bergmann and Fruton synthesized Bz-Tyr-Gly-NHPh from benzoyltyrosine and glycine anilide in the presence of α-chymotrypsin.[14] Obviously these studies were inspired by the assumption that in living organisms the protein biosyntheses would be governed by proteases[15] and previously mentioned plastein reactions having provided a stimulus to hypotheses of that kind. However, as the chemical nature of the plasteins had not been exactly described at that time; it was suggested by Bergmann, that the experimental conditions should be basically simplified in order to accomplish unequivocally definable results. For this reason the authors employed — as it is common practice in peptide synthetic chemistry — partially protected starting compounds to enable an exact product analysis. (For these experiments it did not matter that the derivatized products obtained by the enzymatic approach could not be transformed to the free dipeptides.)

The following quotations emphasize how much the synthetic work of Bergmann and in particular of Fruton (for a review see Reference 16), who played a prominent role in these studies in Bergmann's laboratory, was influenced by the idea that the proteases were the protagonists of the in vivo protein synthesis. Bergmann assumed that "... the proteinases owe their existence to the preexistence of other proteinases. There is in life, a practically endless sequence of reactions, in which one proteinase synthesizes the next by a predetermined reaction, and so forth"[15] further to quote Fruton: "The fact that proteolytic enzymes exhibit all these properties in vitro makes it more likely that they play a central role in the course of in vivo synthesis of proteins."[17]

However, the concept of protein biosynthesis by a simple reversal of enzymatic proteolysis, generally accepted as valid until the end of the 1930s, was questioned by thermodynamic data on peptide bond hydrolysis provided by Borsook and Huffman.[18] These authors showed that, under physiological conditions, the synthesis of peptide bonds represents a strongly endergonic process. In fact the formation of a peptide bond requires an energy input of 2 to 4 kcal/mol, and in 1953 Borsook[19] concluded: "Peptide bonds cannot be synthesized to any significant extent merely by mass action reversal of hydrolysis." Furthermore, when in 1941 Lipmann[20] and Kalckar[21] pointed to the salient role of phosphorus compounds such as ATP as energy sources in biochemical processes, it was suggested that the formation of peptide bonds in vivo might proceed by means of "activated" amino acids. Kalckar for instance found: "There is reason to believe that peptide formation in tissues is always coupled with oxidoreduction just like phosphorylation."[21]

Of the various hypotheses promoted during this period to explain protein biosynthesis, the so-called template theory was most popular. According to this view, the activated amino acids align themselves along specific sites of a polynucleotide template and react together resulting in the assembly of a protein. However, the concept of protease-controlled protein biosynthesis was still not dead. As late as 1955, Fruton stated: "It is possible, that our speculations about protein formation are too simple, whether we assume a polynucleotide template or the coupled synthetic action of proteinases."[22]

The demise of the concept of protein biosynthesis via reversible, protease-mediated hydrolysis was finally brought about by the recognition of the genetic code and the crucial roles played by m- and t-RNAs during the process of in vivo protein synthesis (for more details see, for instance, References 23 and 24). After elucidation of the mechanisms of the ribosomal protein biosynthesis, interest in enzymatic peptide synthesis largely waned. However, it spectacularly revived during the second half of the 1970s with the prospect of the utilization of proteases for preparative scale peptide synthesis (*vide infra*).

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