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BRUCE MERRIFIELD

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A Biographical Memoir by

ULF RAGNARSSON

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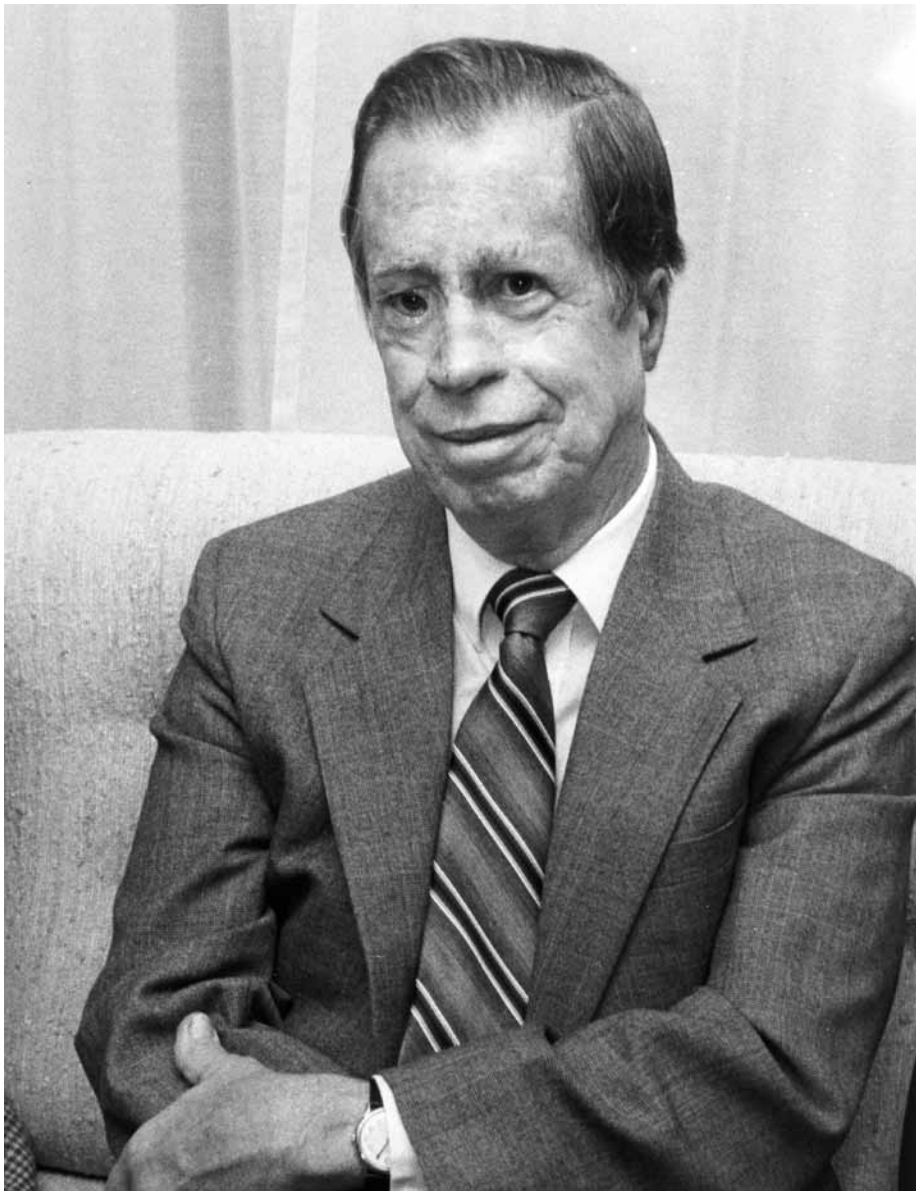

BRUCE MERRIFIELD

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BY ULF RAGNARSSON

BRUCE MERRIFIELD, John D. Rockefeller Jr. Professor Emeritus at Rockefeller University and winner of the 1984 Nobel Prize in Chemistry, was a prominent scientist with major influence on the development of peptide and protein synthesis, a field of great importance during the second part of the twentieth century. His name is associated with a fundamentally novel scientific method that he conceived, pioneered, and, during the rest of his life, further improved. Under his direction, this method was applied to problems of immense complexity.

Bruce was born in Fort Worth, Texas, the only son of George Evan Merrifield, an interior decorator and furniture salesman, and Lorene Lucas Merrifield. The family went to Southern California in 1923 and moved a number of times during the Great Depression. As a result, Bruce attended eleven schools before

high school. He described this period of his life briefly in his autobiography: "The Depression surely affected my life and influenced my future behavior. Stability and security became important, but wealth and social position were not. Steady, hard work and conservative approaches to problems seem to have been superimposed on whatever genetic traits were present."

As a young boy, Bruce enjoyed all sorts of things, including playing with his chemistry set and learning photography, developing his own film, and making prints. His favorite subjects in high school were chemistry, physics, and astronomy, and he had a special inclination toward experimental activities.

He graduated from Montebello High School, in East Los Angeles, in 1939. From there, he first went to Pasadena Junior College for two years and then went to the University of California, Los Angeles (UCLA), where he enrolled as a chemistry major. In the university's new chemistry department, he had outstanding teachers. Bruce recalled afterward that he especially enjoyed courses in qualitative organic analysis and biochemistry. He received his bachelor of science degree in 1943.

His undergraduate biochemistry teacher, Dr. Max Dunn, was an expert on amino acids and introduced Bruce to the topic. At this time, amino acids were still rather exotic compounds. Two of those occurring in proteins were not isolated until after World War I, and it would take about a decade until proteinogenic amino acids of high quality would become commercially available.

After a year performing routine tasks as an animal technician, Bruce decided to go to graduate school, so he returned to UCLA in 1944 and began his thesis research under Dunn. At that time, microbiological procedures were used to assay many commonly occurring substances. Much of Bruce's thesis project seems to have been of microbiological nature; however, his graduate project provided him with unique experiences working with biopolymers and their building blocks.

More specifically, the work involved substances essential for the growth of various *Lactobacillus* species, with the aim to apply these bacteria for quantitative analysis, especially of amino acids. That was an area in which his mentor had pioneered, and Bruce, indeed, succeeded in determining the proline content in two proteins. (It should be pointed out that at this time, no protein structure had yet been characterized at the molecular level, but the characterization of insulin would be completed soon.) In addition, Bruce analyzed the occurrence of pyrimidine bases in yeast and sperm DNA. For this work, he received his PhD in 1949.

Bruce was offered a fellowship with D.W. Woolley at the Rockefeller Institute for Medical Research, in New York City, shortly before graduation. Woolley was known for his wide interests in biologically active chemical

compounds such as vitamins, growth factors, antimetabolites, and neurotransmitters.

Before moving to New York, Bruce married Elizabeth "Libby" Furlong, his girlfriend of many years who had also been a graduate student at UCLA.

At Rockefeller, Bruce was assigned to a project dealing with a new bacterial growth factor called strepogenin that Woolley had discovered and now wanted to characterize more fully. The project was to last for several years. Initially, it involved working with a variety of substances, but then it gravitated towards working with partial hydrolysates of proteins that had to be purified and analyzed before they could be assayed.

For these purposes, unique methods had recently been developed by researchers who worked in the same building as Woolley's laboratory at Rockefeller—for example, counter-current distribution by Lyman Craig and amino acid analysis by William Stein and Stanford Moore. Bruce learned and successfully applied their new methods to his own project.

At about this time, Sanger presented the primary structure of insulin, and from this hormone, well-defined fragments could be prepared and investigated. In particular, two peptides from its B-chain, a pentapeptide and a heptapeptide that both contain serine, were studied because of their high strepogenin activity.

To prove that the activity was real and not due to an artefact or impurity, Bruce embarked on synthesis of the pentapeptide. Using conventional techniques, he was able to prepare a sample with the same physical properties and, most important, identical growth-promoting activity. Other peptides that he subsequently examined, including oxytocin/vasopressin intermediates, led him to the conclusion that those containing either serine or cysteine were particularly active, whereas the remaining part could vary considerably, as demonstrated with a few additional peptides he made. The threonine analog of the pentapeptide was found to competitively inhibit its strepogenin activity.

By 1950, the methods for peptide synthesis had undergone only moderate changes since the days of Emil Fischer, who pioneered the field. Starting from amino acids with complementary protecting groups at their amino and carboxyl functions, selective peptide bond formation could be achieved and the product purified, usually by crystallization. This was a slow process with high loss of product, and one that often failed. To open the way to elongation of the chain, selective removal of one protecting group was required in order to allow a second cycle to be initiated in an analogous way. After a desired number of such cycles, finally all other protecting groups were removed to give the corresponding free peptide.

Some improvements in this process were introduced during the 1930s, such as the reductive cleavage of protecting groups by sodium in liquid ammonia. The

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versatile benzyloxycarbonyl (Z) group, which allowed Vincent du Vigneaud and his coworkers to synthesize the nonapeptides oxytocin and vasopressin, as well as several analogs, should also be mentioned. This outstanding work was performed at about the time when Bruce entered the field of peptide synthesis, and it took place across the street from his laboratory.

After having practiced peptide synthesis for a few years on his own within the strepogenin project, Bruce began thinking of an alternative, more broadly applicable strategy that could also be applied to large targets. In May 1959, he wrote down a few pages in his laboratory notebook, from which the initial lines below are quoted:

A New Approach to the Continuous, Stepwise Synthesis of Peptides.

> There is a need for a rapid, quantitative, automatic method for the synthesis of long chain peptides. A possible approach may be the use of chromatographic columns where the peptide is attached to the polymeric packing and added to by an activated amino acid, followed by removal of the protecting group and with repetition of the process until the desired peptide is built up. Finally the peptide must be removed from the supporting medium.

Bruce presented his ideas to Woolley, who realized their potential and encouraged him to go ahead with the project and start developing it.

The early phase of this is not very well known, and was only briefly described in Bruce's autobiography. In the 2001 film *Peptide and Protein Synthesis: Origin and Development*, Bruce said he initially believed that it would take three months to develop the proof of concept for the new procedure, but instead it took three years. A large number of problems of both chemical and practical nature had to be solved, and a multitude of experimental parameters needed to be optimized.

As a supporting medium for chemical synthesis, Bruce originally tried cellulose powder. His idea was to esterify the first amino acid to the cellulose OH-groups and simultaneously exclude the amino acid from undergoing further reaction via its carboxyl function, but his attempts in that direction failed. Polyvinyl alcohol and polyacrylamide were also attempted before polystyrene came into focus and was more closely investigated.

Polystyrene functionalized with sulfonic acid groups had been applied by others for ion exchange, but Bruce could not modify such polymers in order to be useful in this context. However, unfunctionalized polystyrene, crosslinked with different amounts of divinylbenzene, was also available and had promising mechanical properties. This option was especially appealing as, with only a low degree of crosslinks, the polymer swelled

greatly in organic solvents. It could also easily be derivatized to provide a handle to attach the first amino acid to the polymer as a kind of benzyl ester. Esters were and still are reliable protecting groups for carboxyl groups, so this new approach marked an important step forward in the synthesis of proteins.

Before 1960, the choice of amino-protecting groups and coupling agents for peptide synthesis was not very large. Among the few protecting groups available, Z was not quite optimal because it requires rather strong acidic conditions to be cleaved and a benzyl ester is not completely stable under such conditions, resulting in some loss of the amino acid on the polystyrene. However, after trying a more acid-labile alternative, Bruce decided to avoid new complications and go ahead with the original choice. Thus, the first Z-protected amino acid was esterified to derivatized polystyrene, whereupon Z was cleaved off with acid and the protonated amino function neutralized with tertiary amine to be ready for elongation with the next Z-amino acid.

To form peptide bonds, Bruce originally had active esters in mind, but even when used in considerable excess, they were not reactive enough to couple to all free amino groups. Fortunately, another suitable and powerful agent, dicyclohexylcarbodiimide (DCCI), had then become available, allowing the chain-elongation cycle to dipeptide to be completed.

By the middle of 1961, most of the required exploratory work involving the choice and separate optimal handling of the solid support, temporary protection of the amino group, and the peptide coupling reagent, had been made and efforts could be directed towards an efficient integration of the gained experiences to make peptides. Resin esterified, as indicated previously, was loaded into a specially designed reaction vessel equipped with a sintered glass filter and a stopcock to allow washing and filtering by suction. All subsequent synthetic steps involving the resin were performed continuously in this vessel, thereby eliminating manipulative losses of material.

The resulting cycle included acidic deprotection of the temporary amino-protecting group of the amino acid esterified to the resin; washing; deprotonation to liberate a free amino function; washing; addition of the next protected amino acid and DCCI for coupling to the just-liberated reactive site; and finally, after DCCI coupling, another washing, with all reagents in excess to guarantee the various steps proceeded essentially quantitatively. Then, new cycles involving the same steps could be carried out successively until the chain of the desired peptide target was completed.

For the first publication describing his new procedure, Bruce chose to make a tetrapeptide. This required a few additional innovations, such as stabilization of the ester bond to the resin, repetition of the coupling step,

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and blocking of unreacted amino functions by acetylation. The completed peptide was cleaved from the resin by saponification. Then, the crude product was carefully purified and simultaneously characterized with respect to the presence of all theoretically possible peptidic side products, later called deletion peptides. This last step involved using ion-exchange chromatography, whereupon the desired peptide was obtained, pure by several criteria and identical with authentic reference material.

Bruce's publication of this process established solid phase peptide synthesis (SPPS) as a promising alternative to the older methods of peptide synthesis. Although the underlying principles were questioned by a few members of the peptide community, the paper attracted enthusiastic attention among chemists worldwide and was soon followed by even more spectacular work. A very large number of the more than 300 papers that Bruce eventually published dealt with developments and applications of SPPS, of which only a few can be commented on here.

Shortly after, the tetrapeptide paper was followed by publications dealing with the synthesis of the nine-residue peptide bradykinin. This work featured application of the newly developed t-butoxycarbonyl (Boc), instead of Z, and incorporation of side-chain-protected three-functional amino acids, as well as a generally optimized technique, allowing the elongation rate of the peptide chain to be increased to two residues per day. Starting from resin loaded with the COOH-terminal amino acid, formation of eight peptide bonds, cleavage of the product from the support and purification to biologically fully active bradykinin could then be accomplished in eight working days.

The rate could be increased to about six residues per day using an automatic synthesizer, which was constructed in close cooperation with John Stewart, also a coworker of Woolley. Subsequently, Stewart made a very large number of bradykinin analogs and other peptides by automated synthesis.

In addition to the reaction vessel, the automatic synthesizer prototype was composed of components to store, select, and transfer the required reagents, and a stepping drum programmer controlled all of the steps. Although this instrument was described in detail, within a few years, less robust commercial models appeared on the market. Some of them, unfortunately, discredited SPPS severely.

Until around 1965, SPPS was a one-man show, but within the next couple of years, Bruce was joined in his efforts by both guest investigators and students, allowing work to expand into the angiotensin, insulin, oxytocin, and peptide antibiotic fields. With the arrival in 1967 of Bruce's first postdoctoral fellow, Bernd Gutte from Germany, the ambition was raised to the synthesis

of a protein with enzymatic activity, ribonuclease A, composed of no less than 124 amino acids.

The completion of this landmark project required an incredible 369 chemical reactions and 11,931 steps by the automatic synthesizer, without isolation of any intermediates. After rigorous purification, it provided a product with, substrate specificity, analytical and kinetic profiles in very close agreement with those of the natural protein, as judged from amino acid analysis, enzyme digestion, antibody neutralization, peptide mapping of a tryptic digest, paper electrophoresis, gel filtration, and ion-exchange chromatography. Furthermore, it exhibited a specific activity of 78 percent towards yeast RNA.

This work furnished experimental support for Anfinsen's assertion that the primary structure of a protein determines its tertiary structure, and it drew attention to SPPS from a wider audience than before, including the world of protein chemists and enzymologists. Thus, Moore and Stein wrote in the Rockefeller University Annual Report for 1968–1969, page 18: "Enzyme chemistry in 1969 took a major step forward with the synthesis of bovine pancreatic ribonuclease. The chemical structure of the protein had been elucidated in the preceding decade by Stanford Moore and William Stein and their associates on the fifth floor of Flexner Hall; the chemical synthesis of the enzyme by Bruce Merrifield and Bernd Gutte on the fourth floor of Flexner Hall brought this subject full circle."

Bruce, on the same page of the report, summarized the work as follows: "During the past year this laboratory has continued to concentrate on the solid phase methods for the chemical synthesis of peptides. Our most exciting advance was the synthesis of ribonuclease."

Parallel with more work being done with SPPS, it also became evident that there were side-reactions and other imperfections that occasionally gave rise to experimental difficulties, and that, therefore, further improvements in methodology were required. Beginning around 1970, Bruce systematically reinvestigated essentially every component and step in his procedure with the aim to improve it, from the ester link to the resin, to the relative stabilities of temporary amino- and side-chain-protecting groups, the coupling efficiency and its measuring and monitoring, and the final cleavage of the peptide chain from the support with hydrofluoric acid (HF). Quantitative measurements and criteria were required and often became routine. These efforts further consolidated the procedure.

The loss of peptide from the original resin, which occurred also on cleavage of Boc with acid, totalled 83 percent, corresponding to an average of 1.4 percent per step in the ribonuclease synthesis. To avoid this, the Pam-resin was introduced. In addition to exhibiting about 100 times greater acid stability, it was found to

completely eliminate another problem encountered earlier: the undesirable formation of a peptide family with permanently blocked amino functions, which was generally very difficult to remove from the desired target in the final purification step.

Various handles on the polymer, allowing cleavage under very mild acidic conditions or at alternative sites after the peptide chain had been completed, were also introduced and increased the scope of SPPS. After purification, large segments prepared on resins such as those mentioned can be used to make provision for protein synthesis by chemical ligation.

Further attention was given to the properties of the protecting groups used. This was a field that, in the meantime, had grown immensely. The selectivity of benzyl-based protection of side-chain OH-, COOH-, and NH_2 groups to acid was improved, resulting in reduced amounts of subsequent premature cleavage and subsequent side-reactions at these sites.

In model studies, the efficiency of various coupling conditions was investigated and, using mass spectroscopy, it could be shown that the amount of deletion and insertion peptides could be reduced to a remarkable 0.03 to 0.04 percent under optimal conditions with the Pam-resin, corresponding to a stepwise yield of more than 99.9 percent. For the final cleavage of the product from the resin, liquid HF had been introduced early and applied in the synthesis of ribonuclease. Although useful in most instances in this context, cases of severe damage by this reagent gradually accumulated. As a consequence, an alternative milder S_N2 HF procedure was developed in the Merrifield laboratory that could be applied separately or as a first step in combination with the earlier method.

In possession of the improved SPPS methodology, Bruce then returned to synthesis of biologically active peptides on an extended scale. With different aims in mind, a very large number of peptides, such as glucagon, gastrin, cecropin, melittin and melittin hybrids, thymosins, and immunoglobulin fragments (most of them composed of less than 50 amino acids) were successfully synthesized.

Particular attention was paid to the antibacterial cecropin and to the 29-residue hormone glucagon that, together with insulin, is responsible for the regulation of the glucose level in blood. After a reliable synthesis for glucagon had been worked out, within a project spanning more than ten years, a very large number of glucagon analogs were systematically made. In this way, a detailed knowledge of the influence of several individual amino acids on the hormonal activity was determined. This work also lead to increasingly powerful glucagon antagonists that were the prerequisite to further investigations of pharmacological and biological nature, beautifully exemplifying the power of SPPS in structure-activity studies of peptide hormones.

In 1982, Bruce and his coworkers were the first to synthesize an antibacterial cecropin peptide, which then opened the field to systematic characterization of this class of compounds. Cecropin A is a 37-residue peptide of insect origin that belongs to a group of closely related substances involved in the immunological response to bacterial infections. After the structures of the major cecropins had been confirmed by synthesis, a few analogs were prepared and examined for antibacterial activity against various pathogenic bacteria.

As an additional feature, the all-D-cecropin A was synthesized and found to generate not only the same electrical conductivity as its enantiomer in lipid bilayers, but also equal activity against representative bacteria. Cecropins become highly helical in nonpolar media. These and analogous results with enantiomers of two additional antibiotics allowed Bruce to conclude that these peptides exert their action by formation of ion-channels in cell membranes without interaction with chiral species like enzymes or receptors.

Today, the strongly increasing interest in all sorts of biologically active peptides requires efficient methods for their synthesis and further studies. Since about 1975, work concerned with peptide synthesis has increasingly been based on application of SPPS, especially work on a moderate scale aimed at total synthesis, structure-activity studies, and the provision of material for biological investigations, but also work on a considerably larger scale

Nobel laureates in chemistry posed for this group photo after the 1991 Nobel Lectures. From left are: Jerome Karle, Sidney Altman, Yuan T. Lee, Johann Deisenhofer, Hartmut Michel, Bruce Merrifield, Richard R. Ernst, Herbert C. Brown, Dudley R. Herschbach. At far right is physics laureate Hans G. Dehmelt, who was absent when the physics photo was taken.

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in the pharmaceutical industry. Early on, solid-phase methods impacted nucleic acid synthesis and, more recently, organic chemistry in general (SPOC). Last, but not least, it should be pointed out that SPPS allows an individual scientist to conveniently prepare peptides that formerly required large teams of highly trained peptide chemists working over long periods of time.

Bruce received many awards and honors, including the Lasker Basic Medical Research Award, the

Gairdner International Award, the American Chemical Society Award for Creative Work in Synthetic Organic Chemistry, the American Peptide Society Alan E. Pierce Award, and the European Peptide Society Josef Rudinger Memorial Lecture Award, and he was elected as a member of the National Academy of Sciences in 1972. He was awarded the Nobel Prize in Chemistry in 1984 "for his development of methodology for chemical synthesis on a solid matrix." Besides these accomplishments, he received fifteen honorary doctoral degrees.

Bruce was survived by his wife Elizabeth and their six children—Nancy, Jim, Betsy, Cathy, Laurie, and Sally—as well as sixteen grandchildren.

For more details about Bruce's personal and scientific life and his interactions with members of his research group at the Rockefeller University and colleagues around the world, the reader of this memoir is referred to his autobiography, *Life During a Golden Age of Peptide Chemistry* (American Chemical Society, 1993), and the special issue in 2008 of the journal *Peptide Science* (volume 90, pages 153-477), which was dedicated to his memory and contains his complete bibliography.

Bruce Merrifield is remembered as an unassuming, friendly, and stoic man, a generous and inspiring mentor and colleague, and an optimistic and painstaking scientist who had great intuition. He was a person to look up to, a role model of a scientist, and a key player in the golden age of peptide chemistry.

Author's note: I was an early guest investigator with Bruce Merrifield and stayed in close contact with him thereafter. I thank David Eaker, a former colleague; Alexander R. Mitchell, a former post doc; and Svetlana Mojsov, a former graduate student of Bruce Merrifield, for generous information and comments. My final and rather special thanks to Mrs. Elizabeth Merrifield for the privilege to author this memoir.

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